LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-TR-639673

Year 4 Report Multiplex Assay Development for Detection of Potential Bioterrorism Agents in Food Matrices

P. Naraghi Arani

June 21, 2013

Disclaimer

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

LLNL-TR-

Year 4 Report

Multiplex Assay Development for Detection of Potential Bioterrorism Agents in Food Matrices

Pejman Naraghi-arani
Naraghiarani2@llnl.gov
Lawrence Livermore National Laboratory

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

Year 4 Report

Multiplex Assay Development for Detection of Agents with Bioterrorism Potential in Food

Lawrence Livermore National Laboratory

Executive Summary:

An 18-plex assay has been developed for the simultaneous detection of *Bacillus anthracis, Yersinia pestis, Francisella tularensis, Brucella abortus, Brucella melitensis, and Salmonella enterica*. The assay contains three signatures (ie: primer/probe triplets) for the detection of *Bacillus anthracis* (one signature targeting the chromosome of the agent, one signature targeting pXO1, and one signature targeting pXO2), four signatures for the detection of *Yersinia pestis* (one signature targeting the chromosome and one signature targeting each of 3 plasmids), two signatures for the detection of *Francisella tularensis* (both chromosomal signatures), one signature for *Brucella abortus* (targeting the chromosome), two signatures for *Brucella melitensis* (targeting the chromosome), and two signatures for the detection of *Salmonella enterica* (both signatures target specific virulence genes in virulent *Salmonella enterica* strains). The assay includes a positive control signature targeting an externally added template (*Erwinia herbicola* genomic DNA), a negative control signature targeting *Thermotoga maritime* [Tm] (a bacterium found in deep ocean vents), an instrument control signature consisting of the Tm probe with Cy-3 moieties attached to one bead class, and a fluorescence control consisting of a Tm probe with multiple biotin moieties attached to one bead class.

Three genomic templates per agent were used for testing the *Yersinia pestis*, *Francisella tularensis*, *Bacillus anthracis,* and *Brucella abortus/Brucella melitensis* signatures to ensure that the new assay is still able to detect these pathogens. The assay is able to detect at least 0.1pg genomic DNA/reaction for *Yersinia pestis,* at least 0.1pg genomic DNA/reaction for *Bacillus anthracis,* at least 0.1pg genomic DNA/reaction for *Francisella tularensis,* at least 1pg genomic DNA/reaction for *Brucella abortus,* and at least 0.1pg genomic DNA/reaction for *Brucella melitensis*. For *Salmonella enterica* signature testing, a total of 5 genomic templates were tested and the multiplex was able to detect at least 0.1pg genomic DNA/reaction.

Four candidate *Salmonella enterica* signatures were incorporated into the previous version of the assay (Panel 3) and tested to select the best signatures to integrate. Two of the four signatures were removed for high backgrounds and cross-reactivity within the multiplex. The remaining two signatures were incorporated into the new "Panel 4" assay to move forward with for further testing.

Limit of detection (LOD) with a confidence interval of 95% or greater for each signature by copy number (determined using artificially generated template nucleic acids incorporated into each of 15 plasmids and spiked in combination into each reaction) is summarized in Table 1.

Assay sensitivity and selectivity studies concluded that the assay can provide confirmatory detection (i.e.: multiple assay signatures targeting different sections of agent genome generating Median Fluorescence Intensities [MFI] above threshold) for the vast majority of target genomes tested.

In tests exploring potential cross-reactivity with over 200 background and near-neighbor templates at high spikes, two near-neighbor templates were found to cross-react with both newly incorporated *Salmonella enterica* signatures. *Salmonella enterica salamae (*MZ1440) showed that it will cross react with both *Salmonella* signatures (S1 and S2) in the multiplex. *Salmonella enterica houtanae* IV (MZ1442) also displayed a very weak cross reactivity with one of the two *Salmonella* signatures (S1) within in the multiplex as well.

Two other signatures, one targeting *Yersinia pestis* [Yp1 (Yp2040)] and one targeting *Francisella tularensis* [Ft1 (Ft9941)] also demonstrated some cross-reactivity with various soils, eukaryotes, and microbes which are detailed in the Cross-Reactivity Study Results section.

Table of Contents

Executive Summary	Page 3
Limit of Detection Summary Table	Page 5
Materials and Methods	Page 6
Results	Page 8
Summary of Signature History	Page 9
Thresholds	Page 10
Limit of Detection on Whole Agent Targets	Page 11
Multi-Strain Test Results	Page 18
Cross-Reactivity Study Results	Page 22
Limit of Detection on Artificial Templates	Page 26
Conclusions	Page 41

Table 1

Per-Signature Limit Of Deteciton (Minimum 95% Confidence Interval)

Signature	Average MFI	MFI Range	Threshold	% Above Threshold	≥95% Reactive	LOD (copies/rxn)
Yp1 Genomic (2040)	171.08	3320 - 5400	761	100 (20/20)	Y	1000
Yp2 pCP1 (4683)	5.13	38 - 424	50	95 (19/20)	Y	1000
Yp3 pMT1 (5554)	15.20	260 - 761	50	100 (20/20)	Y	1000
Yp4 pCD1 (8989)	4.20	53 - 464.5	50	100 (20/20)	Y	1000
Ft1 Genomic (9941)	28.50	2368 - 4618	50	100 (20/20)	Y	100
Ft2 Genomic (9983)	8.94	129 - 784.5	50	100 (20/20)	Y	100
Ba2 pX01 (6115)	6.08	19 - 579	50	100 (20/20)	Y	1000
Ba1 Genomic (6132)	4.63	44 - 353	50	100 (20/20)	Y	1000
Ba2 pX02 (9485)	13.38	250.5 - 1529	50	100 (20/20)	Y	100
Bru1 Genomic (9958)	3.90	280 - 1007	50	100 (20/20)	Y	100
Bru2 Genomic (9969)	6.95	147 - 570	50	100 (20/20)	Y	100
Bru3 Genomic (9970)	5.73	96 - 1132	50	100 (20/20)	Y	100
S1 (prgI_v2_3)	13.08	267 - 1013	50	100 (20/20)	Y	1000
S2 (ssaV_v2_10)	10.98	194 - 431.5	50	100 (20/20)	Y	1000

Table 1: Signature names are listed with their genome target regions and the LLNL-assigned signature number in order to facilitate cross-referencing. Tests were conducted using multiple plasmids, each containing the target region of each signature, simultaneously spiked into reactions and titrated over 8 logs. Each reaction was repeated 20 times and a signal above threshold (Thresholds set using a minimum of 700 data points with a 2% maximum false positive rate per signature) in at least 19 of 20 cases indicated an LOD point with a confidence interval of 95% or greater.

Furthermore, tests on high concentrations of total nucleic acid extracts from 32 soil samples collected from across the continental United States, 45 bacterial samples taken from Lawrence Livermore National Laboratory (LLNL) stocks as well as those purchased from the American Type Culture Collection (Manassas, VA) and other vendors, 16 eukaryotic organisms (purchased from various vendors), and 11 cell culture extracts (generate at LLNL) demonstrated minimal cross-reactivity with these materials (see Cross-Reactivity Study Results section). It is anticipated that with guidance from FDA CFSAN, additional tests including those conducted in food matrices may also be appropriate, or the FDA CFSAN may choose to conduct its own cross-reactivity tests once the assay panel becomes available to them.

Materials and Methods (General Description):

All reactions were carried out on 96 well PCR plates (Accuflow, E&K Scientific, Santa Clara, CA) in a total volume of 25µL (20µL master mix plus 5µL sample) optimized for multiplexed PCR. A volume of 20µL PCR master mix (Maxima Hot Start PCR Master Mix (2x) – 500 reactions, Cat# K1052, Thermo Fisher Scientific Biosciences, Lafayette, CO) contains: 12.5µL 2X PCR Master Mix, 3µL 15-plex primer mix (0.4 mM final concentration each forward and reverse primer), 0.5µL *Erwinia herbicola* DNA (positive control) at 2.5 pg/reaction, and 4µL of PCR grade water (Ambion, Austin, TX). Forward primers were biotinylated with a single biotin moiety at the extreme 5' end of each primer while Reverse primers were unmodified. Probes had a C-18 spacer at the 3'end with a carboxyl moiety at the end of the C-18 spacer. All oligonucleotides were purchased as high purity reagents (90% or better purity guarantee) from BioSearch Technologies, Novato, CA.

The contents of the 15-plex Master Mix (15 Biotinylated Forward primers and 15 standard Reverse primers) are listed below.

Maxima Hot Start PCR Master Mix: 12.5µL/reaction

15-plex Primer mix: 3µL/reaction (400uM final concentration per primer)

Erwinia herbicola (positive control): 0.5µL/reaction

PCR grade water: 4µL/reaction

Sample: 5µL/reaction

The cycling parameters for the assay are summarized in Table 2 below:

Table 2
FDA-Biosearch Thermocycling Parameters

Cycle	Repetitions	Temp.	Duration	
1	1	95ºC	4 Min.	
2	35	95ºC	30 Sec.	
		60ºC	30 Sec.	
		72ºC	1 Min.	
3	1	72ºC	2 Min.	
4	1	4ºC	HOLD	

For *Yersinia pestis*, *Francisella tularensis*, *Erwinia herbicola*, *Salmonella enterica* , and *Bacillus antrhacis*, total nucleic acid extracts were prepared using the "Master Pure" total DNA extraction kit purchased from Epicentre Technologies (Epicentre Technologies, Madison, WI) as per manufacturer instructions. For *Brucella abortus* and *Brucella melitensis*, genomic DNA was ordered from the Department of Defense Critical Reagents Program (CRP) (Maryland, USA).

Master mixes were prepared inside of a PCR Workstation (AirClean® Systems AC600 PCR Hood Series, Raleigh, NC). Master mix material was loaded on to 96-well PCR plates and transferred to a second PCR Workstation for template addition and sealing of plates. Sealed plates were then spun at 1000 rpm in a plate centrifuge for one minute in order to ensure complete addition of any reagents stuck to sides of plate wells and transferred to a thermocycler (MJ Research-Now BioRad Inc., Hercules, CA). PCR cycling was carried out as shown in Table 2. Upon completion of thermal cycling, plates were removed and spun a second time as described to reduce potential for cross-contamination of wells by condensate droplets adhering to film.

Following PCR amplification, 5µL of PCR product was added to 22µL of microbead mixture. Unconjugated beads supplied by Luminex Corp. (Austin, TX). The amplified product was denatured at 95°C for 2 minutes, hybridized at 55°C for 5 minutes, and immediately removed from the 96-well heated thermocycler. Following hybridization, 100µL of heated (55˚C) Tris-NaCl buffer was added to the hybridized oligo-bead reactions and this mixture was transferred to a pre-wetted 96-well filter bottom plate, 1.2µm pore size (Millipore Corp., Billerica, MA). The hybridized oligo-bead reactions on the filter plate were washed twice with heated (55˚C) 100 µL Tris-NaCl to remove unhybridized nucleic acids. The PCR products bound to the beads were labeled by adding 60µL SAPE (Streptaviden R-phycoerythrin conjugate 3 ng/µL (Moss, Inc; Pasadena, MD) and were incubated in the dark for 5 minutes. Beads were washed once with heated (55˚C) 100 µL Tris-NaCl, re-suspended in 100µL Tris-NaCl and analyzed using Bio-Plex 100® (Bio-Rad Laboratories, Hercules, CA) instrument.

Results:

As previously reported (see Year 1 and Year 3 reports) and as shown in Table 3, from an initial pool of 43 pathogen-specific signatures, 13 were down-selected for incorporation into the multiplex panel. For the introduction of *Salmonella enterica* signatures to Panel 3, 2 signatures were down-selected for incorporation into the multiplex panel from an initial pool of 4 signatures targeting virulence factors found in virulent *Salmonella enterica* strains, as shown in Table 3.1. Four factors eliminated a signature from consideration for incorporation into the multiplex: High MFIs when tested on water blank samples (see High Backgrounds in Table 3); Cross-reactivity with one or more other agents; lack of reactivity, or poor reactivity with target templates. Although each of these signatures performed well in singleplex reactions, upon incorporation into the multiplex panel, these issues were observed with two of the four signatures. The down-selected signatures incorporated into the multiplex panel did not suffer these limitations.

S1 (prgI_v2_3) and S2 (ssaV_v2_10) are two signatures targeting virulence genes in virulent *Salmonella enterica* strains. Both signatures have a granular LOD of 1000 copies/reaction when tested against synthetic plasmid templates. One issue we have run across with the multiplex was during the background and near neighbor testing. Both S1 and S2 showed reactivity against *Salmonella enterica salamae (*MZ1440). S1 showed weak reactivity against *Salmonella enterica houtanae* IV (MZ1442). It is suspected (but not tested) that these two strains both carry the prgI gene and that *Salmonella enterica salamae* (MZ1440) also carries the ssaV virulence gene. Yp1 (Yp2040) and Ft1 (Ft9941) signatures showed some cross reactivity (2 of 3 or 3 of 3 replicates) with 5 soil samples, while the S1 signature showed some cross-reactivity (2 of 3 replicates) with *Bacillus subtilis* . The data is summarized on tables 15-16.1 of this report.

One other potential issue is with Yp2 (Yp4683). This signature's response showed a decrease in MFI compared with Panel 3 data with the switch to the Thermofisher Maxima enzyme. While the granular LOD remained 1000copies/reaction, the overall endpoint MFI response of the signature has changed. The signature is still within acceptable limits of detection.

Table 3

Summary of Signature History

Organism	Target	Signature	Elimination Criteria			Final Result (Pass or Fail elimination criteria)
			High Backgrounds	Cross Reactivity	Lack of Reactivity	
F. tularensis	Genomic	0000				FAIL
		0012				FAIL
		0051				FAIL
		9941				PASS
		9983				PASS
B. anthracis	Genomic	6133				FAIL
		6126				FAIL
		6132				PASS
		6135				FAIL
		6144				FAIL
		6147				FAIL
		6201				FAIL
	PA gene (pX01)	6116				FAIL
		6118				FAIL
		6130				FAIL
		6125				FAIL
		6115				PASS
		6120				FAIL
	PA gene (pxX02)	9469				FAIL
		9489				FAIL
		9472				FAIL
		9478				FAIL
		9485				PASS
		9496				FAIL
Y. pestis	Genomic	1994				FAIL
		1996				FAIL
		2006				FAIL
		2007				FAIL
		2008				FAIL
		2009				FAIL
		2034				FAIL
		2036				FAIL
		2003				FAIL
		2010				FAIL
		2011				FAIL
		2014				FAIL
		2018				FAIL
		2040				PASS
	pPMT1	5554				PASS
		5564				FAIL
		5574				FAIL
		5577				FAIL
	Pls gene (PCP1)	4698				FAIL
		4705				FAIL
		4683				PASS
	pCD1	9078				FAIL
		9147				FAIL
		8989				PASS

B. abortus	**Genomic**	9913				**See Comment**
		9914				**See Comment**
		9915				**See Comment**
		9916				**See Comment**
		9917				**FAIL**
		9954				**See Comment**
		9956				**See Comment**
		9958				**PASS**
		9961				**FAIL**
		9962				**FAIL**
		9963				**FAIL**
		9964				**See Comment**
		9965				**FAIL**
		9966				**See Comment**
B. melitensis	**Genomic**	9906				**See Comment**
		9969				**PASS**
		9970				**PASS**
		9971				**See Comment**
S. enterica typhi	**Virulence Gene Targeted**	prgI_v2_3				**PASS**
		prgI_primux2				**FAIL**
		ssaV_v2_10				**PASS**
		ssaV_v2_14				**FAIL**

Two signatures targeting virulence genes found in *Salmonella enterica* were selected for incorporation into the multiplex. While two candidate signatures were eliminated due to high backgrounds and cross-reactivity.

Thresholds:

Thresholds for discrimination of positive from negative signals were set using over 700 negative data points for each signature. Data from tests conducted using various targets, near-neighbors, and background nucleic acid extracts were compiled into receiver operating characteristic (ROC) curves. A ROC curve is a graphical plot of the sensitivity or true positive rate vs. false positive rate for a binary classifier system such as the one we are trying to develop here to discriminate positive from negative signals.

Median Fluorescence Intensities (MFIs) corresponding to a positive signal were calculated for each signature based on the compilation of signals from a minimum of 1000 negative reactions. A negative reaction was defined as any test where the template for that particular signature was not present. Thus, for each Yp targeting signature, all background extracts such as soils, prokaryotes, and eukaryotes as well as target tests using Ft or Ba templates were considered as negative reactions. From the compiled signals from over 700 negative reactions, MFIs were assigned using only the 95% upper confidence interval for MFIs at the 98th percentile value. Thus, signature thresholds were set such that a false positive signal would not be expected in greater than 2% of all tests using this multiplex assay. Furthermore, we can say with confidence in excess of 95% that we will never see a false positive rate for any of the signatures in this multiplex panel greater than 2%. The thresholds thus generated are listed in Table 4.

Table 4
Signature Threshold Values
(All MFI Values Rounded to the First Whole Integer)

Signature	MFI @ 98th Percentile Value	95% Upper confidence Interval for MFI @ 98th Percentile Value	Threshold MFI Used in 16-Plex Assay
Ft1 Genomic (9941)	11	14	**50**
Ft2 Genomic (9983)	10	12	**50**
Ba 1 Genomic (6132)	8	11	**50**
Ba 2 pX01 (6115)	9	10	**50**
Ba 3 pX02 (9485)	9	11	**50**
Yp1 Genomic (2040)	601	761	**761**
Yp2 pCP1 (4683)	11	14	**50**
Yp3 pMT1 (5554)	11	14	**50**
Yp4 pCD1 (8989)	11	13	**50**
Bru1 Genomic (9958)	21	28	**50**
Bru2 Genomic (9969)	13	15	**50**
Bru3 Genomic (9970)	12	14	**50**
S1 (prgI_v2_3)	20	28	**50**
S2 (ssaV_v2_10)	10	13	**50**

The thresholds used to determine LOD were conservatively rounded up to an MFI of 50 (unless already above an MFI of 50) to prevent the threshold from being too low as to be seen as normal background.

Please note that as additional data is generated using this multiplex assay, these thresholds can be reset at the users' discretion in order to increase or decrease sensitivity with concomitant increase or decrease in false positive rates respectively in order to accommodate case for use.

Limits of Detection on Whole Agent Targets:

Initial limit of detection determinations were conducted using total nucleic acid extracts from one strain of each target organism in triplicate over an 8-log titration spanning 10pg/reaction to 1attogram/reaction. Titration curves (MFI vs. a log-titration series of decreasing amounts of genomic template) for each of the three agents are shown in Figures 1-6. As each signature represents an independent PCR reaction, each signature has a threshold that is set independently as observed from Figures 1-3 and Table 4. This is significant as it means that there is no significant cross-hybridization or cross-reaction between

signatures in the multiplex. Furthermore, please note that all 15 amplification reactions (3 Ba signatures, 4 Yp signatures, 2 Ft signatures, 3 Bru signatures, 2 *Salmonella enterica* signatures, and 1 positive control signature) are occurring simultaneously. Thus, Figures 1-6 report target signature titrations separately in order to make graphical representation less complex. In fact, Figures 1-6 represent data from the same set of reactions. Please note that some thresholds overlap one another and are not clearly visible on the plotted graphs.

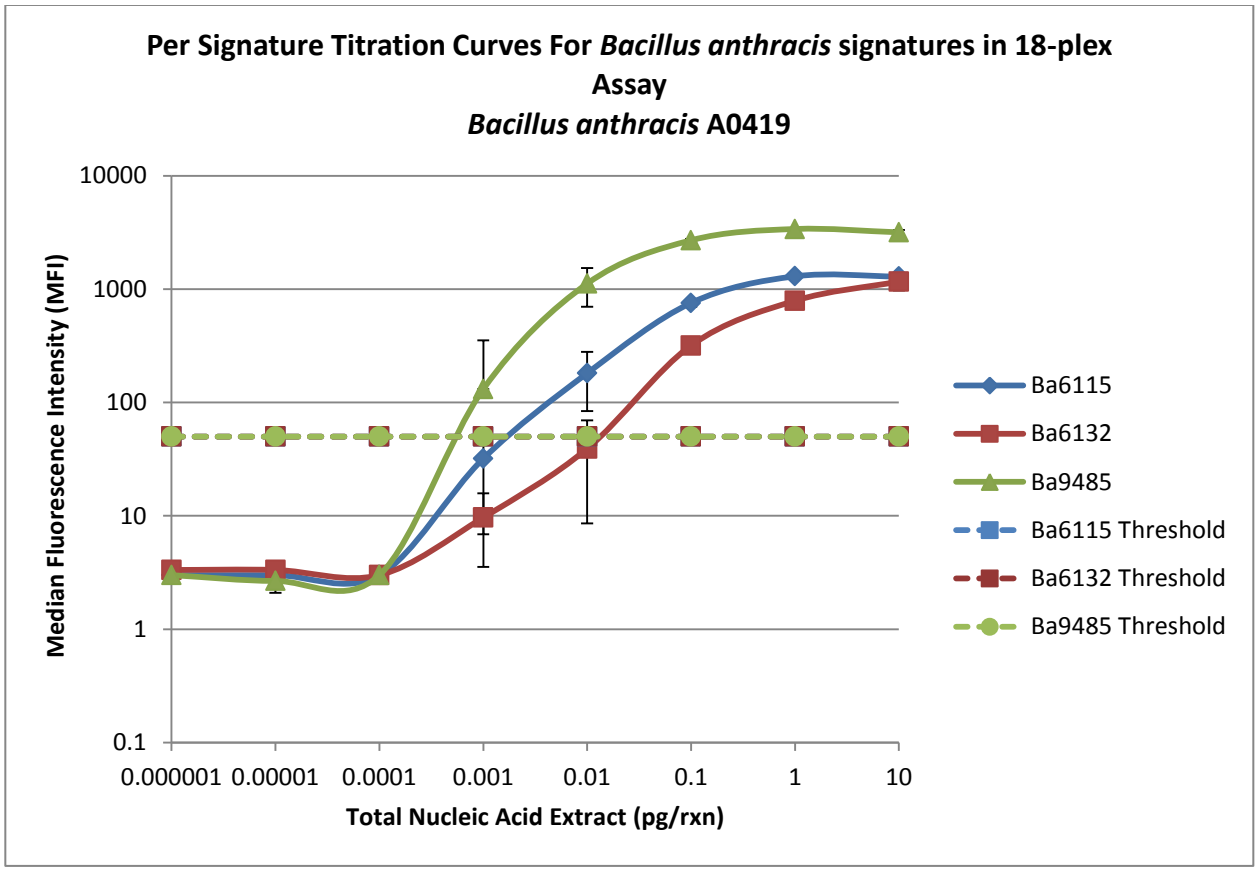

Fig. 1: Titration of total nucleic acid extract from *Bacillus anthracis* strain A0419 assayed by the multiplex assay. Template was diluted in water from 2pg/µL to 0.2ag/µL. Five ul of each dilution was added to multiplex PCR reactions in triplicate. Dotted lines represent each signature's individual threshold. Solid lines represent Ba1 signature response, Ba2 signature response, and Ba3 signature response. Data with standard deviation bars are shown.

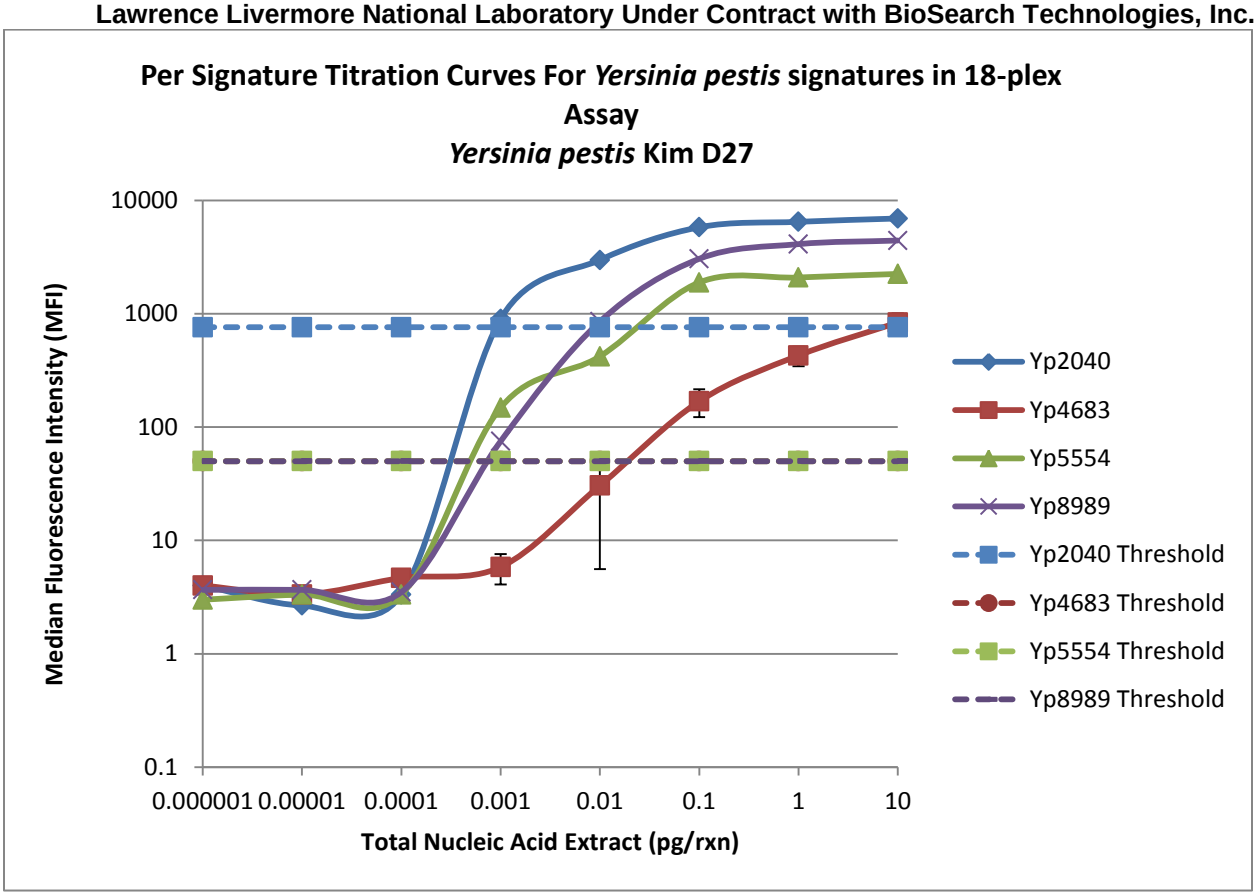

Fig. 2: Titration of total nucleic acid extract from *Yersinia pestis* strain Kim D27 assayed by the multiplex assay. Template was diluted in water from 2pg/µL to 0.2ag/µL. Five ul of each dilution was added to multiplex PCR reactions in triplicate. Dotted lines represent each signature's individual threshold. Solid lines represent Yp1 signature response, Yp2 signature response, Yp3 signature response, and Yp4 signature response. Data with standard deviation bars are shown.

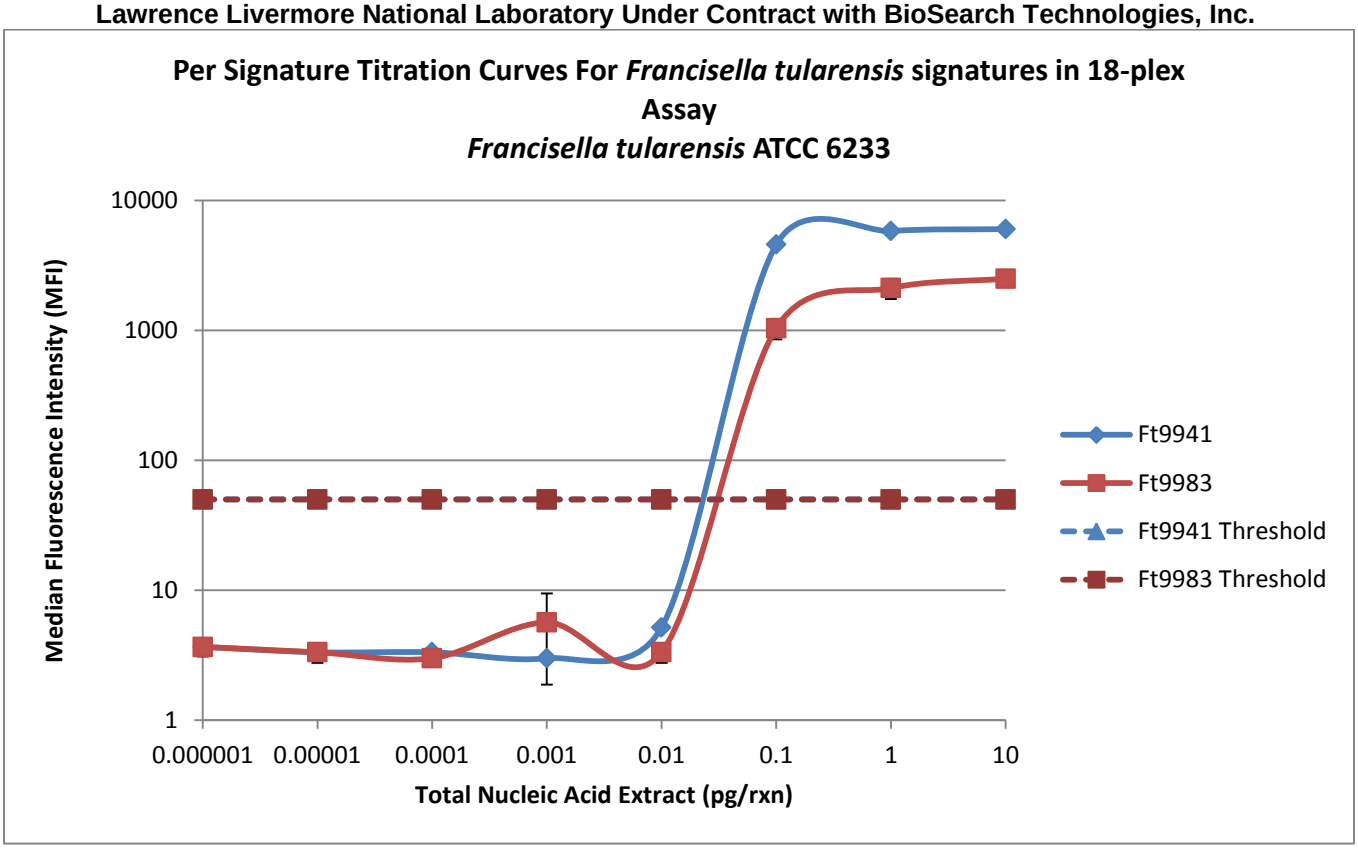

Fig. 3: Titration of total nucleic acid extract from *Francisella tularensis* strain Ft ATCC 6233 assayed by the multiplex assay. Template was diluted in water from 2pg/µL to 0.2ag/µL. Five ul of each dilution was added to multiplex PCR reactions in triplicate. Dotted lines represent each signature's individual threshold. Solid lines represent Ft1 signature response and Ft2 signature response. Data with standard deviation bars are shown.

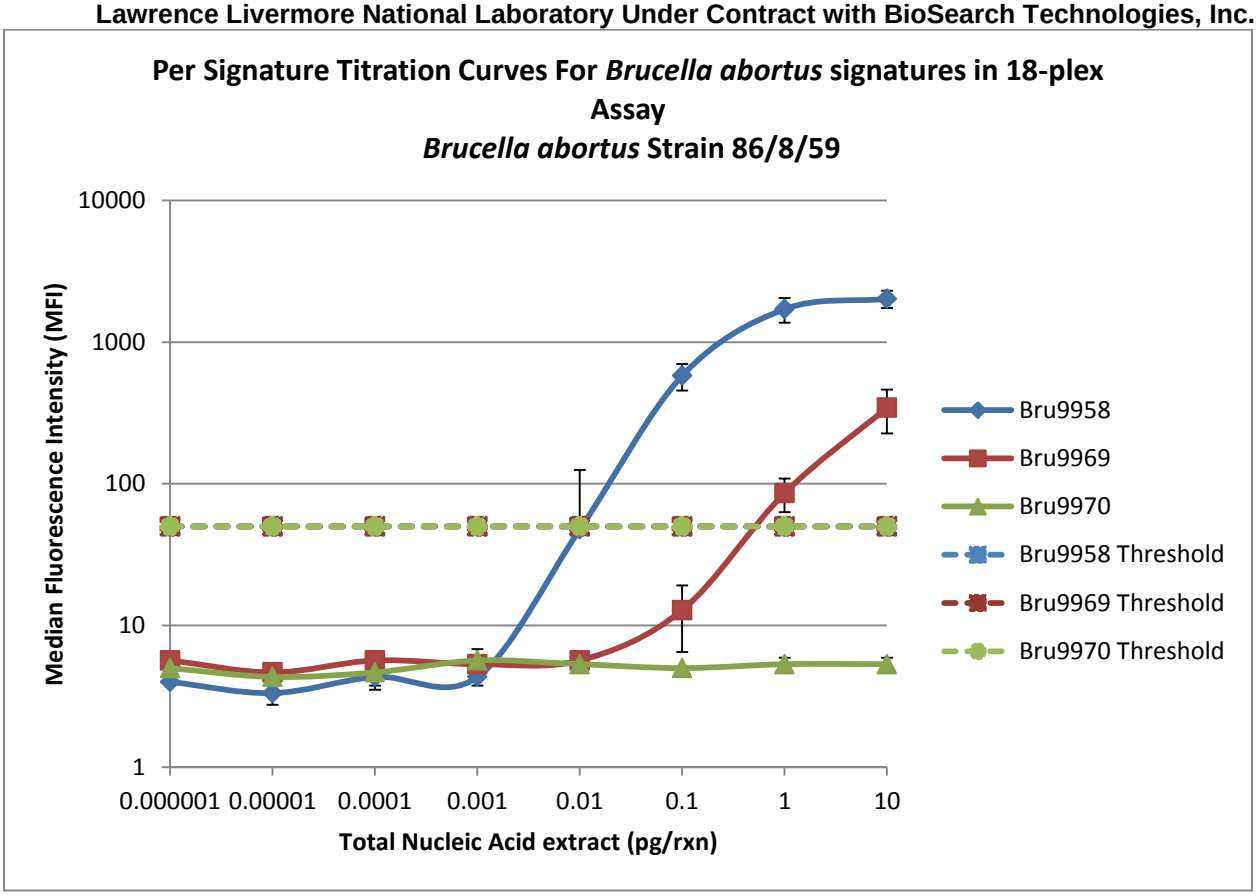

Fig. 4: Titration of total nucleic acid extract from *Brucella abortus* strain 86/8/59 assayed by the multiplex assay. Template was diluted in water from 2pg/µL to 0.2ag/µL. Five ul of each dilution was added to multiplex PCR reactions in triplicate. Dotted lines represent each signature's individual threshold. Solid lines represent Bru1 signature response, Bru2 signature response, and Bru3 signature response. Data with standard deviation bars are shown.

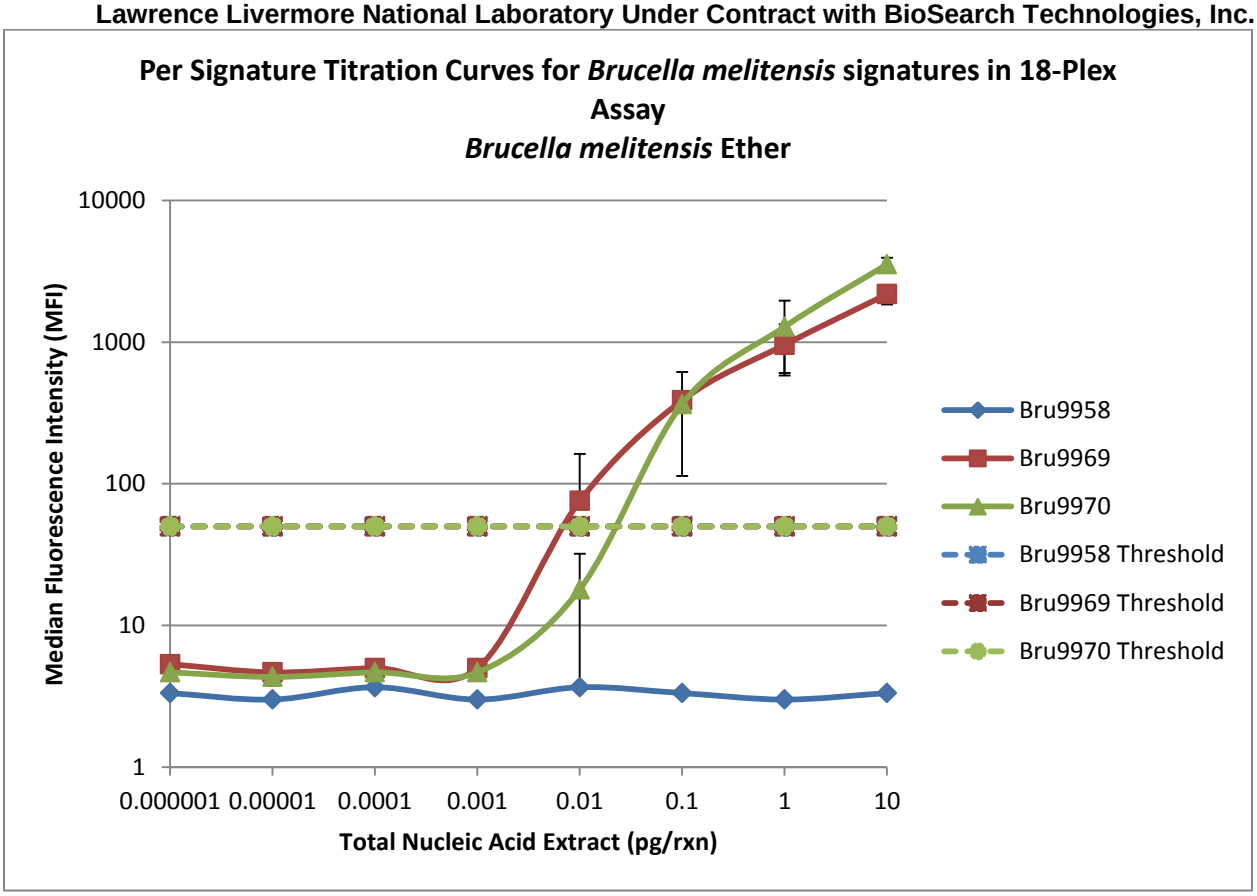

Fig. 5: Titration of total nucleic acid extract from *Brucella melitensis* strain Ether assayed by the multiplex assay. Template was diluted in water from 2pg/µL to 0.2ag/µL. Five ul of each dilution was added to multiplex RT-PCR reactions in triplicate. Dotted lines represent each signature's individual threshold. Solid lines represent Bru1 signature response, Bru2 signature response, and Bru3 signature response. Data with standard deviation bars are shown.

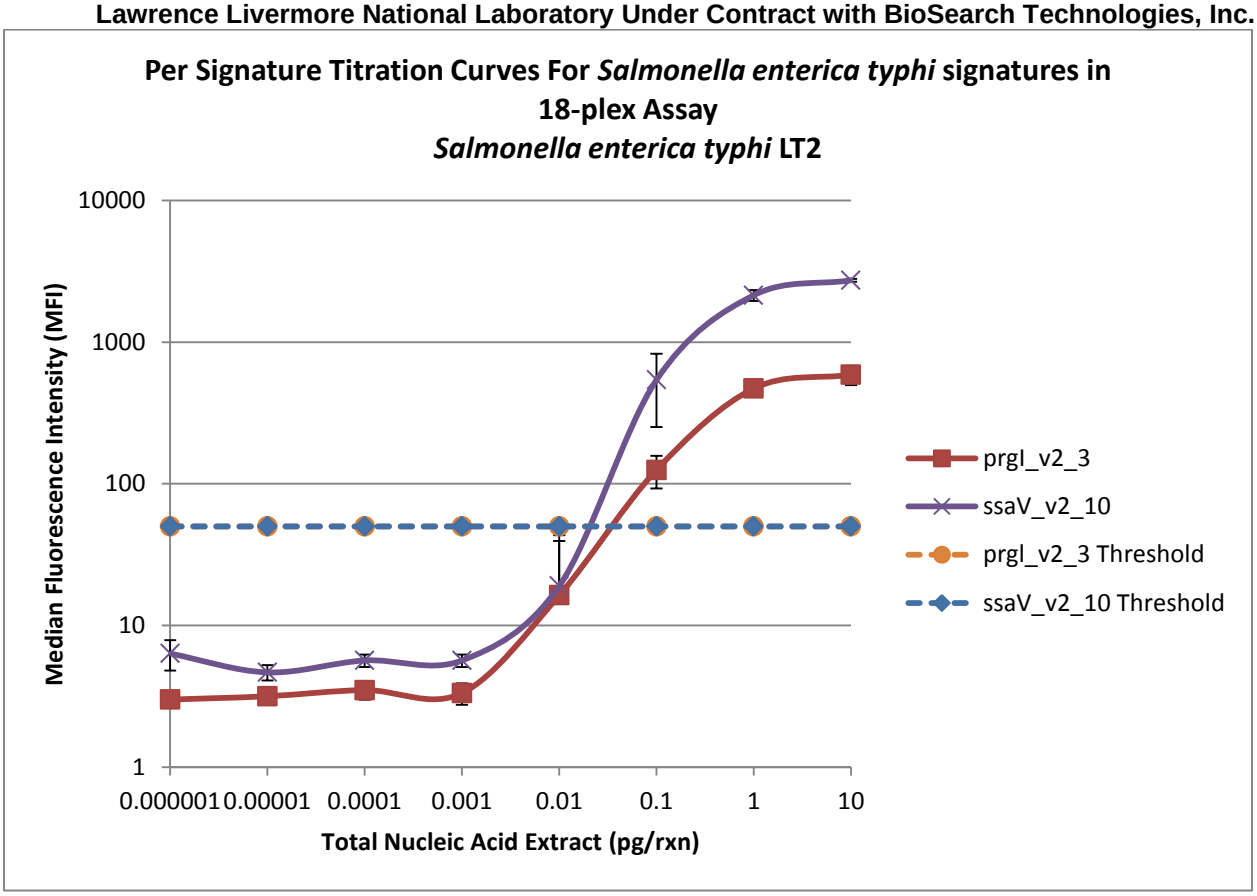

Fig. 6: Titration of total nucleic acid extract from *Salmonella entericaa typhi* strain LT2 assayed by the multiplex assay. Template was diluted in water from 2pg/µL to 0.2ag/µL. Five ul of each dilution was added to multiplex PCR reactions in triplicate. Dotted lines represent each signature's individual threshold. Solid lines represent S1 signature response and S2 signature response. Data with standard deviation bars are shown.

Studies to Determine Assay Applicability to Many Strains of Target Organisms:

In order to test the sensitivity and applicability of the assay for detection of multiple strains of each agent, total nucleic acid extracts from 2 strains of each target agent were tested at 0.1pg/reaction, 1pg/reaction, and 10pg/reaction. The results of those tests are summarized in Tables 5-9. Table 10 shows the 5 different *Salmonella enteria* templates that were tested with the Year 4 multiplex panel.

Table 5
***Bacillus anthracis* Multi-Strain Sensitivity Study**

Target Strain	Target Amount (pg/reaction)	Average MFI Per Signature			Reactive? (Yes/No)
		Ba6115	Ba6132	Ba9485	
Bacillus antrhracis A0297	0.1	995.83	336.67	2663	Yes
	1	1319	870.67	2895.17	Yes
	10	1392.5	1135.83	2817.33	Yes
Bacillus anthracis 0489	0.1	323.83	120	1273	Yes
	1	966.17	561.67	2771.83	Yes
	10	1186.5	1152.33	2674.83	Yes

Table 6
***Yersinia pestis* Multi-Strain Sensitivity Study**

Target Strain	Target Amount (pg/reaction)	Average MFI Per Signature				Reactive? (Yes/No)
		Yp2040	Yp4683	Yp5554	Yp8989	
Yersinia pestis CO92	0.1	4457.5	126.67	1190.5	1841.67	Yes
	1	5760.5	462.33	2283.33	3287.67	Yes
	10	6704	717	2479.33	4417.5	Yes
Yersinia pestis India	0.1	4134.17	57.33	502.5	907	Yes
	1	5897.67	174.67	1336.5	2301	Yes
	10	6600.17	545.5	2196.5	3271.33	Yes

Table 7
***Francisella tularensis* Multi-Strain Sensitivity Study**

Target Strain	Target Amount (pg/reaction)	Average MFI Per Signature		Reactive? (Yes/No)
		Ft9941	Ft9983	
Francisella tularensis LVS ATCC 29684	0.1	4445.67	1029.67	Yes
	1	5763.83	2066.5	Yes
	10	5605.5	2171.5	Yes
Francisella tularensis ATCC 15843	0.1	3621.67	831	Yes
	1	5105.67	1900.17	Yes
	10	5512.17	1905.5	Yes

Table 8
***Brucella abortus* Multi-Strain Sensitivity Study**

Target Strain	Target Amount (pg/reaction)	Average MFI Per Signature			Reactive? (Yes/No)
		Bru9958	Bru9970	Bru9969	
Brucella abortus 86/8/59	0.1	578.67	5.00	12.83	Yes
	1	1706.33	5.33	86.00	Yes
	10	2018.33	5.33	344.50	Yes
Brucella abortus 292	0.1	878.00	5.67	16.17	Yes
	1	2027.67	6	189.67	Yes
	10	1920.33	5.33	245	Yes
Brucella abortus Tulya	0.1	76.67	5.33	6.33	Yes
	1	690.17	6.00	13.5	Yes
	10	1585.67	5.00	131	Yes

Table 9
***Brucella melitensis* Multi-Strain Sensitivity Study**

Target Strain	Target Amount (pg/reaction)	Average MFI Per Signature			Reactive? (Yes/No)
		Bru9958	Bru9970	Bru9969	
Brucella melitensis 63/9	0.1	3.67	157.33	140.50	Yes
	1	3.67	1594.50	1161.83	Yes
	10	3.67	3148.50	2011.67	Yes
Brucella melitensis Ether	0.1	3.33	365.00	388.17	Yes
	1	3.00	1282.50	957.00	Yes
	10	3.33	3533.50	2177.50	Yes
Brucella neotomae 5K33	0.1	3.50	609.67	13.67	Yes
	1	3.67	2386.00	67.17	Yes
	10	3.00	4334.00	386.67	Yes

Table 10
***Salmonella enterica* Multi-Strain Sensitivity Study**

Target Strain	Target Amount (pg/reaction)	Average MFI Per Signature		Reactive? (Yes/No)
		prgI_v2_3	ssaV_v2_10	
S. enterica typhi BAA-639D	0.1	677	540.33	Yes
	1	1989.17	1246.33	Yes
	10	2681.33	1722.83	Yes
S. enterica Paratyphi B SPB7	0.1	367.67	95.83	Yes
	1	1466.67	333.33	Yes
	10	2499.67	534.33	Yes
S. enterica typhi Paratyphi A	0.1	455	235.67	Yes
	1	1692.33	837.17	Yes
	10	2624.83	1328.5	Yes
S. enterica MZ1436	0.1	909.67	225.67	Yes
	1	2328.33	482.33	Yes
	10	2914.67	696.33	Yes
S. enterica MZ1449	0.1	956.5	19.167	No
	1	2210.67	96.67	Yes
	10	2879.17	283.33	Yes

Cross-Reactivity Studies:

In order to determine the potential for the assay to cross-react with environmental nucleic acids, from other prokaryotes, eukaryotes, and near-neighbor organisms, seven panels of total nucleic acid extracts were used in triplicate reactions. For Year 4 near neighbor testing, 10 near neighbors were tested at 100pg/rxn spikes. The panels of templates used and results are summarized in tables 11-16 and results are reported below each table.

Table 11
List of Prokaryotic Organisms Used for Assay Cross-Reactivity Studies

Actinobacillus suis	*Erwina herbicola*	*Porphyrobacter sanguineus*
Aneurinbacillus migulanus	*Escherichia coli*	*Proteus mirabillis*
Bacillus cereus	*Geobacillus caldoxylosilyticus*	*Pseudomonas aeruginosae*
Bacillus globigii	*Halomonas halmophila*	*Pseudomonas oleovorans*
Bacillus subtilis	*Heamophilus influenza*	*Rhizobium leguminosarum*
Bacillus thuringiensis	*Herbaspirillium seropedicae*	*Rhodococcus rhodochrous*
Borrellia burgdorferi	*Lactobacillus garvieae*	*Salmonella typhimurium*
Bifidobacterium denticum	*Lactobacillus gasseri*	*Simonsiella muelleri*
Burkholderia capacia	*Listeria monocytogenes*	*Sphingomonas sp. (Alcaligenes sp)*
Caulobacter vibriodes	*Listeria seeligeri*	*Staphylococcus aureus*
Clavibacter michganensis	*Micrococcus luteus*	*Streptococcus pneumoniae*
Clostridium butyricum	*Moraxella lacunatica*	*Streptomyces scabiei*
Corynebacterium pseudodipthericum	*Oceanospirillium ssp. Maris*	*Tatlockia maceachernii*
Cytophaga marinoflava	*Paenibacillus napthalaenovorans*	*Vibrio paraheamolyticus*
Erwina amylovora	*Paracoccus dentrificans*	*Xanthomonas translucens*

The prokaryotic templates used were a collection of extracts generated at LLNL and other templates purchased from commercial vendors. Each test contained 100pg of the total nucleic acid extract spiked into each reaction. All signals for all of the signatures were reproducibly below established thresholds for all templates in this panel.

Table 12
List of Eukaryotic Organisms Used for Assay Cross-Reactivity Studies

Avian (Chicken)	Flea	*Ovine* (Sheep)
Bovine (Cow)	*Homo sapiens Sapiens* (Human)	*Lagomorpha* (Rabbit)
Canine (Dog)	Mosquito	*Rattus* (Rat)
Drosophila melanogaster (Fruit Fly)	*Murine* (Mouse)	*Simian* (Monkey)
Equine (Horse)	*Porcine* (Pig)	Tick
Feline (Cat)		

The eukaryotic templates used were a collection of total genomic nucleic acids purchased from commercial vendors. Each test contained 5ng of the total nucleic acid extract spiked into each reaction. All signals for all of the signatures were reproducibly below established thresholds for all templates in this panel.

Table 13 **List of Total Nucleic Acid Extracts from Soil Samples Used for Assay Cross-Reactivity Studies**				
D000402 #53	D000561-8-6	S259	S282	S296
D000109 #50	D000562-30-5	S260	S283	S297
D000502-12	D000501-14-2	S271	S284	S298
D000500-26-1	D000550-20	S272	S286	S299
D000505-11-4	S251	S273	S287	S300
D000521-23	S252	S274	S288	S301
D000551-5	S253	S275	S289	S303
D000527-3	S254	S276	S290	S304
D000531-21	S255	S277	S291	S305
D000542-6	S256	S279	S292	S307
D000533-17-1	S257	S280	S295	

Total nucleic acid extracts were generated at LLNL from soil samples collected from 54 locations across the United States. These soils had been collected from urban, suburban, and rural environments. Each soil sample is curated with detailed information as to location, time of day, flora and fauna present at the collection site, etc. Each test contained 5ng of total nucleic acid spiked into each reaction. Two signatures (Yp2040 and Ft9941) showed cross-reactivity or random spikes against various backgrounds (soils, microbes, and eukaryotes). Yp2040 was the only signature to show true (3 out of 3) cross reactivity with 3 different soils (S286, S287, and D000405 #53). The new enzyme is more robust in terms of amplification so the cross-reactivity in previous panels may have been present but not over the set threshold for that panel.

Table 14 **Background Soil Cross Reactivity**			
Signature	**Template**	**Mux Pos. Replicates (Avg.MFI)**	**Comment**
Yp2040	S273	1 out of 3 (119)	
	S286	3 out of 3 (1487)	
	S287	3 out of 3 (300)	
	S288	1 out of 3 (198)	
	S303	2 out of 3 (190)	
	S307	1 out of 3 (340)	
	D000402 #53	3 out of 3 (614)	
	D000521-23	1 out of 3 (362)	
	D000561-8-6	1 out of 3 (437)	
Ft9941	S290	1 out of 3 (240)	
	S303	1 out of 3 (697)	
	S305	2 out of 3 (930)	

Table 15 **Background Prokaryotic and Eukaryotic Organisms Cross Reactivity**			
Signature	**Template**	**Mux Pos. Replicates (Avg.MFI)**	**Comment**
Yp2040	*Actinobacillus suis*	1 out of 3 (980)	
	Corynebacterium pseudodiptericum	1 out of 3 (553)	
	Ovine (Sheep)	1 out of 3 (241)	
	Streptomyces scabei	1 out of 3 (982)	
Ft9941	*Streptomyces scabei*	1 out of 3 (512)	
	Tick	1 out of 3 (293)	
S1 (prgI_v2_3)	*Bacillus subtilis*	2 out of 3 (247)	

The Year 4 multiplex panel was tested against 10 Near Neighbor templates as seen below:

Table 16 ***Salmonella enterica* Near-Neighbor Sensitivity Study**				
Target Strain	**Target Amount (pg/reaction)**	**Average MFI Per Signature**		**Comment**
		S1 (prgI_v2_3)	**S2 (ssaV_v2_10)**	
S. bongori 122472	100	5.67	3.00	
S. enterica houtanae IV MZ1442		108.5	3.67	
S. enterica salamae MZ1440		2789.17	733.33	
S. enterica indica MZ1447		11.33	3.33	
Shigella flexneri 24570		4.00	3.33	
E. coli CFT079		4.33	3.00	
E. coli EDL933		4.67	3.33	
E. coli 8739D-5 58304269		5.67	3.33	
E. coli MG1655		7.00	3.33	
E. coli BAA-460D-5 58143874		5.67	3.33	

TABLE 16.1 ***prgI_v2_3 and ssav_v2_10*** **TaqMan EXCLUSIVITY SCREENING RESULTS**					
Organism	**Strain**	**ATCC* ID**	**Number of copies of organism**	**Signature Ct Results**	
				prgI_v2_3	**ssav_v2_10**
Salmonella enterica houtanae (IV)	MZ1442	BAA-1581	19,100	N/A	N/A
Salmonella enterica salamae	MZ1440	BAA-1583	19,100	24.2 (1.0)	25.0 (0.2)

A total of 10 near neighbors were tested against the Panel 4 multiplex. *Salmonella enterica salamae* MZ1440 cross-reacted with both S1 and S2 signatures. This is an indication that the near neighbor used has picked up, at some point, both the S1 and S2 virulence genes. This cross-reactivity was also noted during the TTA1 (Technical Tropic Area 1, Department of Homeland Security) TaqMan development work (Table 16.1). Weak reactivity was noted for S1 against *Salmonella enterica houtanae* IV MZ1441 but not for S2 in the multiplex testing.

In conclusion, actual cross-reactivity (3 out of 3 tests) was observed only for Yp1 (2040) against three different soils (S286, S287, D000402 #53) based on the established thresholds for the Panel 4 multiplex. Random spikes were noted but are not considered true cross-reactive templates as they only spiked once or twice when tested in triplicate. In order to determine whether or not these are true reactives or if there is true cross-reactivity with off-target nucleic acids in the soil, it would be necessary to sequence the amplicons resulting from these reactive samples. Then, if the amplicon generated was the target amplicon, the designation of cross-reactive would not be quite accurate and would mean that the target for the signature is present in these soil samples. However, if the sequences generated were those intended, then further investigation is needed.

Limit of Detection Studies Utilizing Artificial Templates:

In order to determine copy number sensitivity for each signature, multiple plasmids, each containing the full amplicon region of each signature were generated by BioSearch Technologies (Novato, CA), the sequences of those inserts verified, and the plasmids used in combination for our limit of detection studies. Briefly, each plasmid was quantitated using spectrophotometry after linearization with a single cut enzyme. All plasmids for each agent (i.e.: 2 plasmids for Ft, 3 plasmids for Ba, 3 plasmids for Bru, 4 plasmids for Yp, and 2 plasmids for Salmonella) were mixed in equimolar amounts and a titration series conducted for LOD determination. Titration response curves for each signature are shown in Figures 4-12.

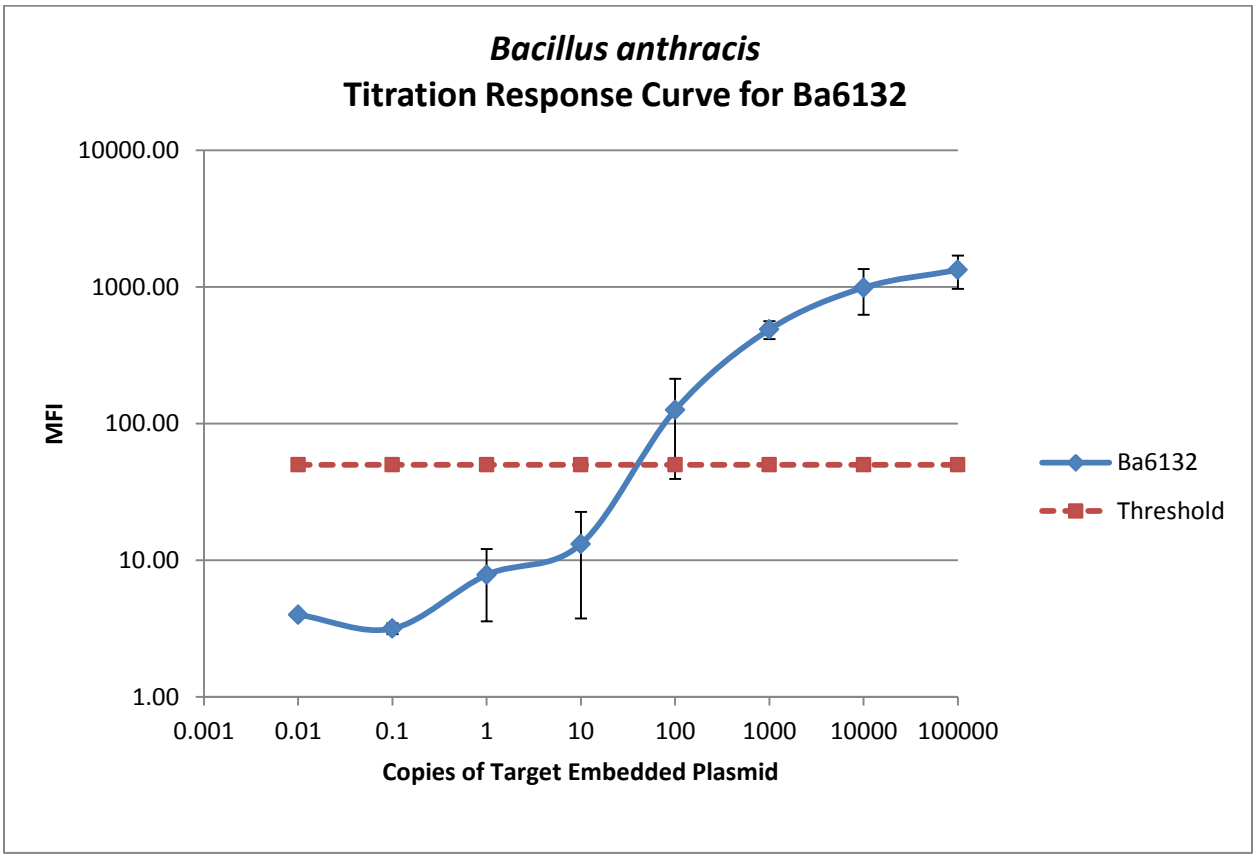

Fig. 6: Titration response curve for Ba1 (6132) signature. Target-embedded plasmids for all *Bacillus anthracis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 6 shows a sensitivity of 100 copies per reaction for the Ba1(6132) signature.

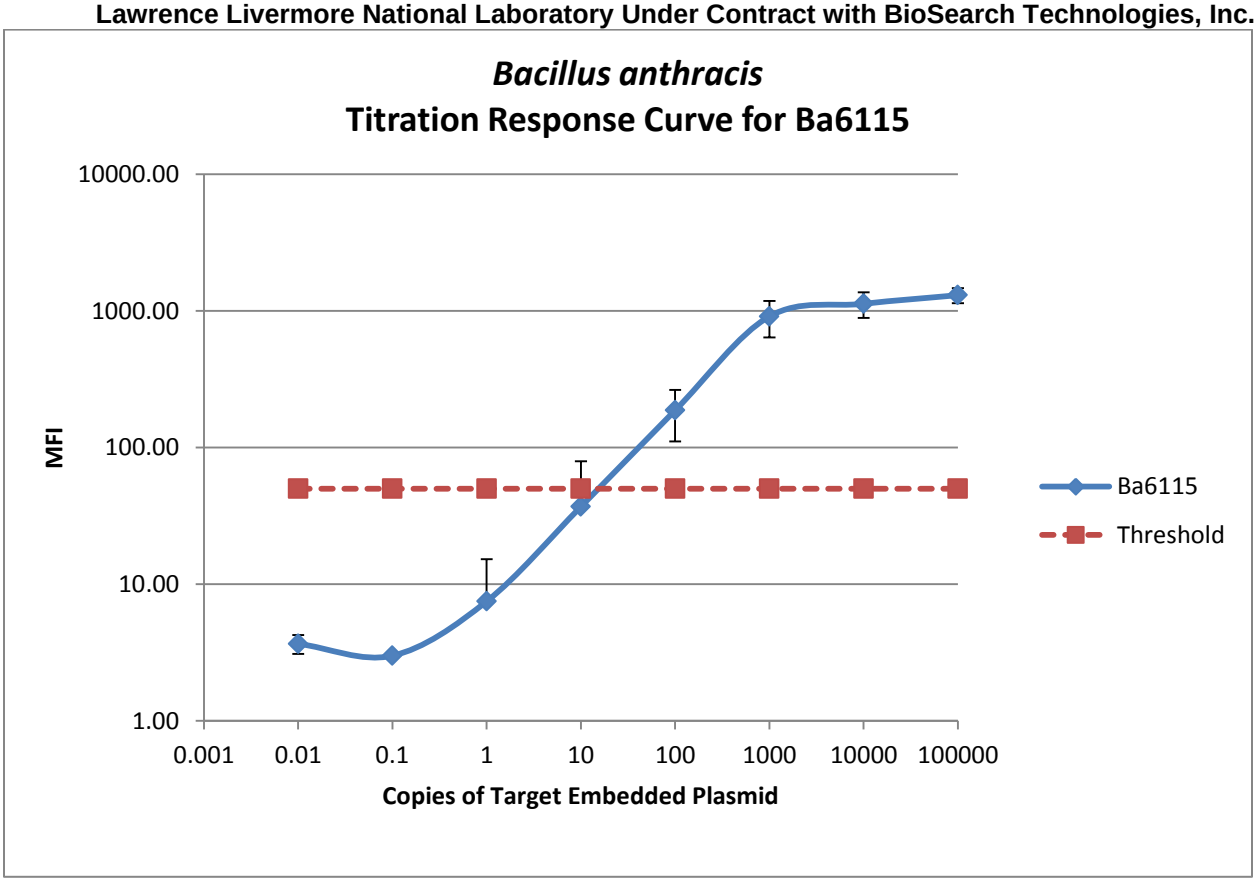

Fig. 7: Titration response curve for Ba2 (6115) signature. Target-embedded plasmids for all *Bacillus anthracis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 7 shows a sensitivity of 100 copies per reaction for the Ba2 (6115) signature.

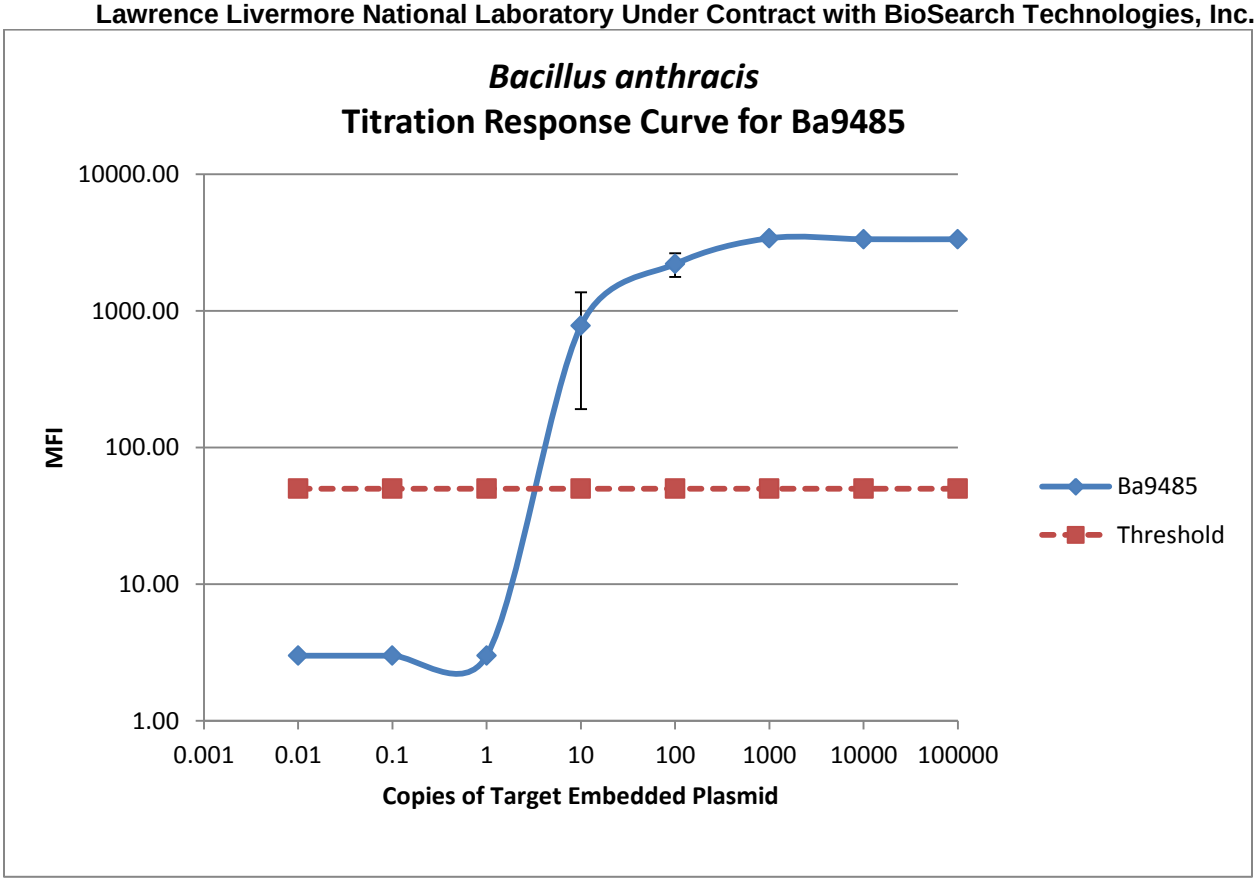

Fig. 8: Titration response curve for Ba3 (9485) signature. Target-embedded plasmids for all *Bacillus anthracis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 8 shows a sensitivity of 10 copies per reaction for the Ba3(9485) signature.

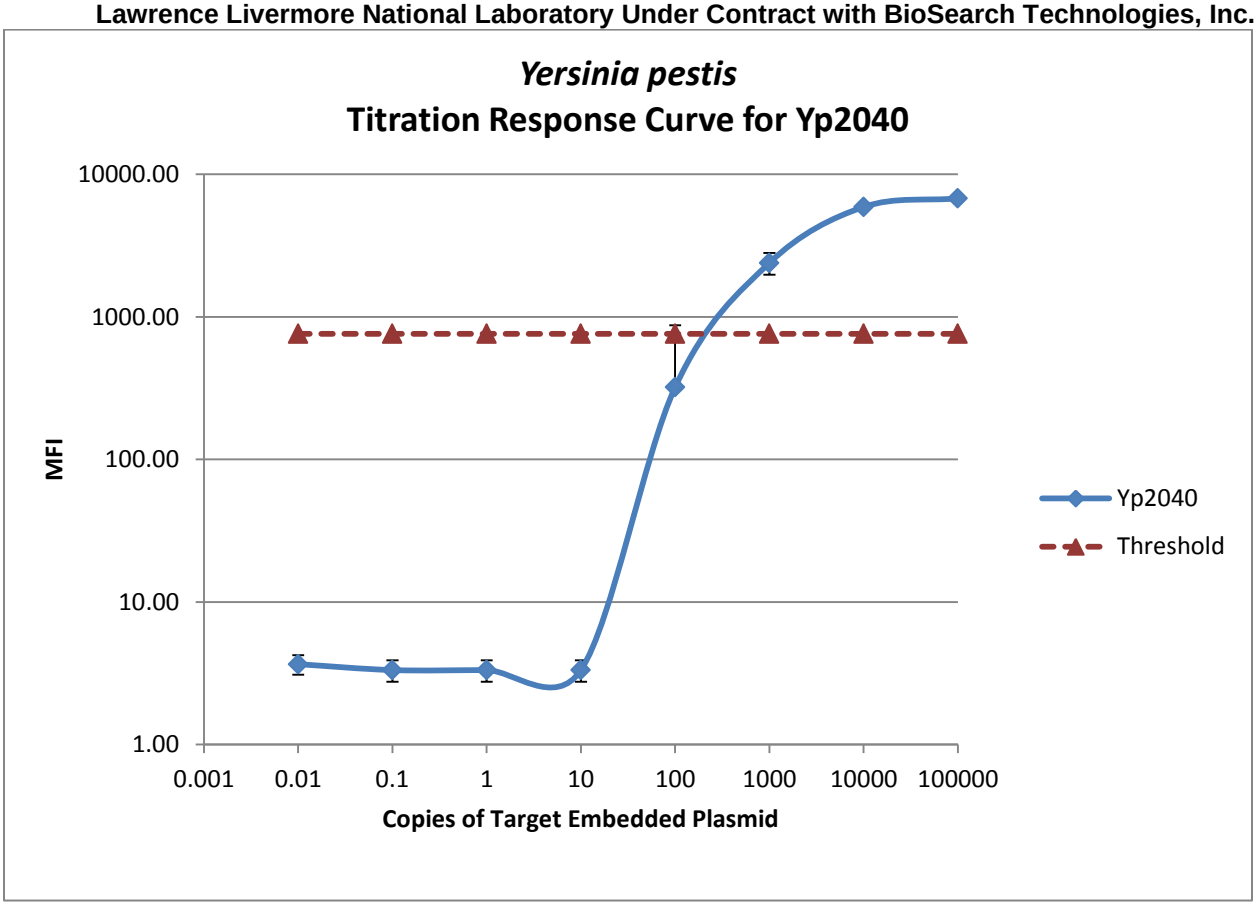

Fig. 9: Titration response curve for Yp1 (2040) signature. Target-embedded plasmids for all *Yersinia pestis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 9 shows a sensitivity of 1000 copies per reaction for the Yp1(2040) signature.

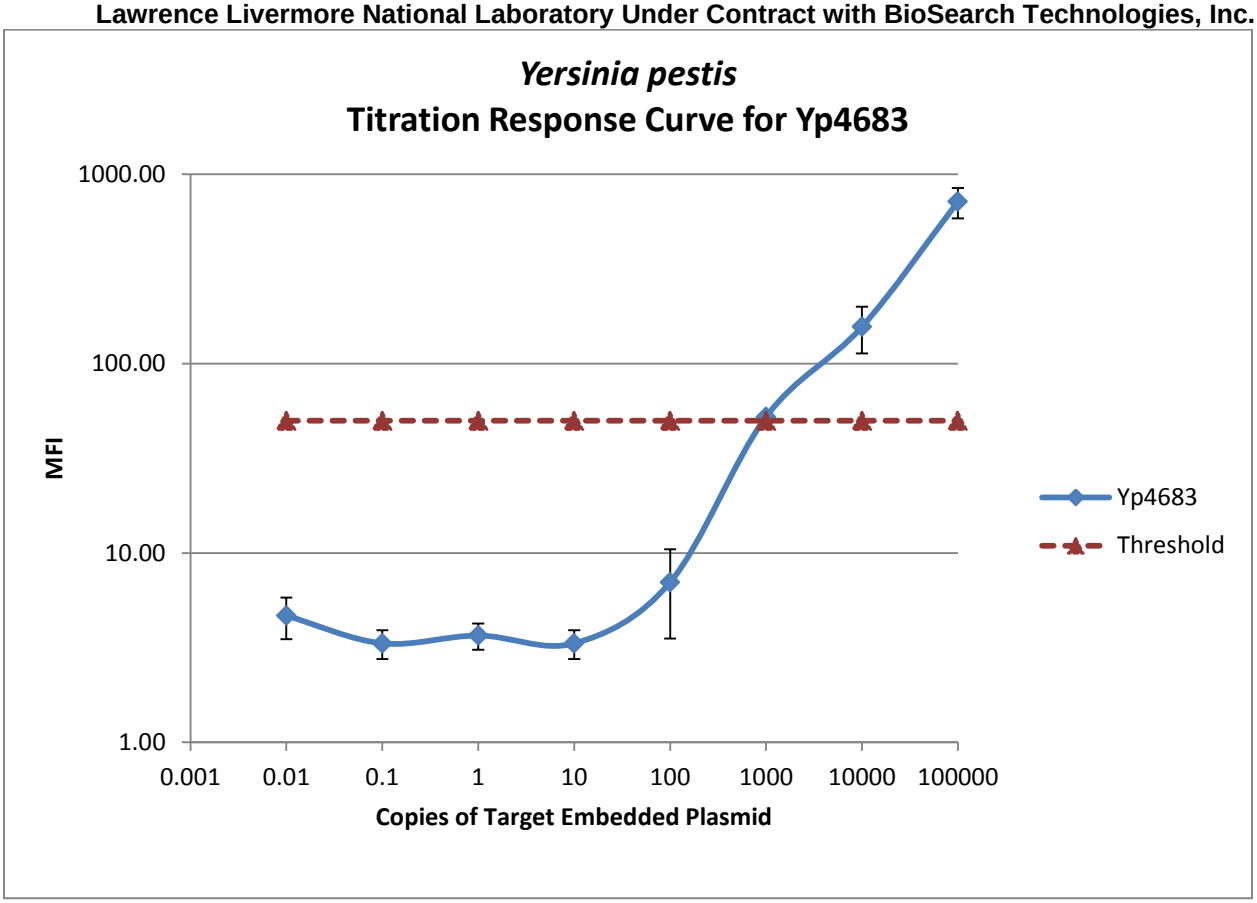

Fig. 10: Titration response curve for Yp2 (4683) signature. Target-embedded plasmids for all *Yersinia pestis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 10 shows a sensitivity of 1000 copies per reaction for the Yp2 (4683) signature.

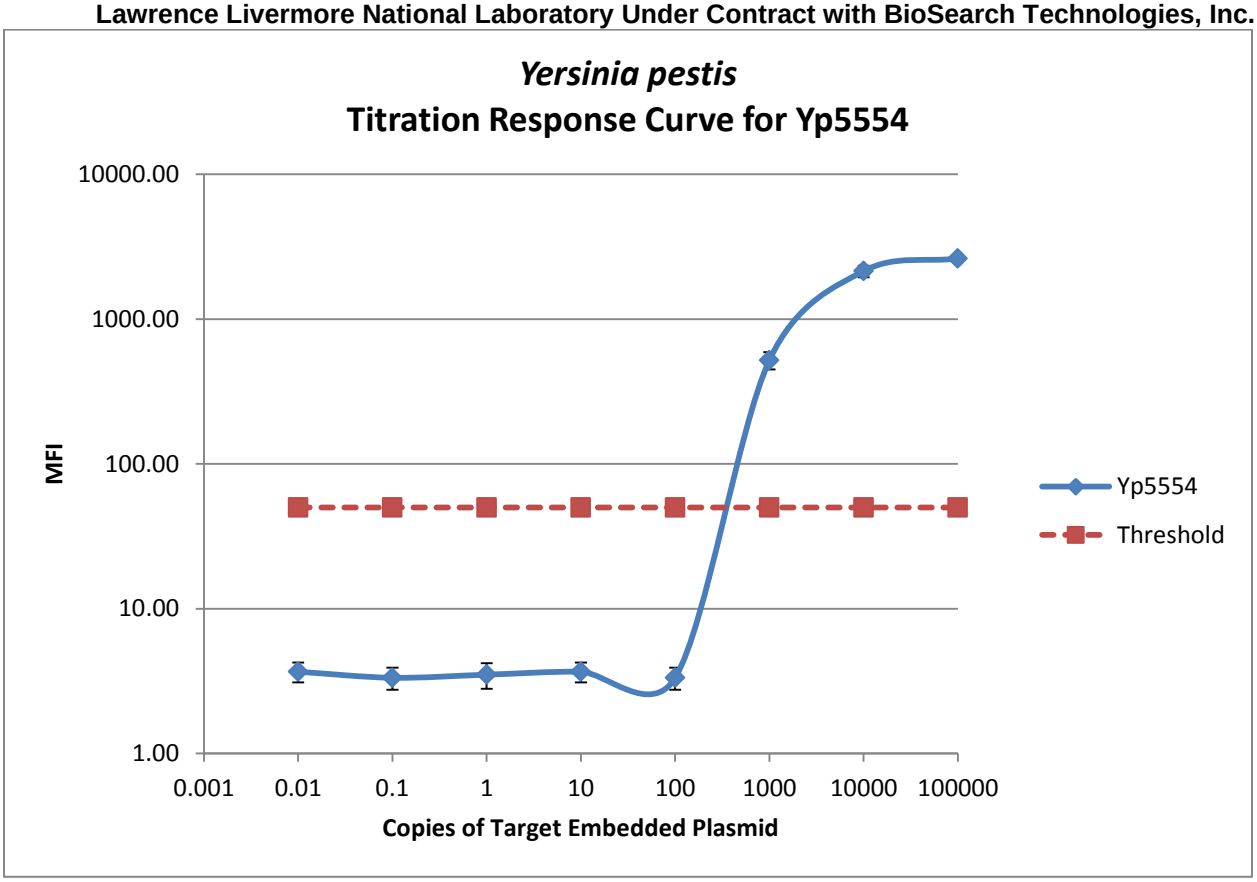

Fig. 11: Titration response curve for Yp3 (5554) signature. Target-embedded plasmids for all *Yersinia pestis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 11 shows a sensitivity of 1000 copies per reaction for the Yp3 (5554) signature.

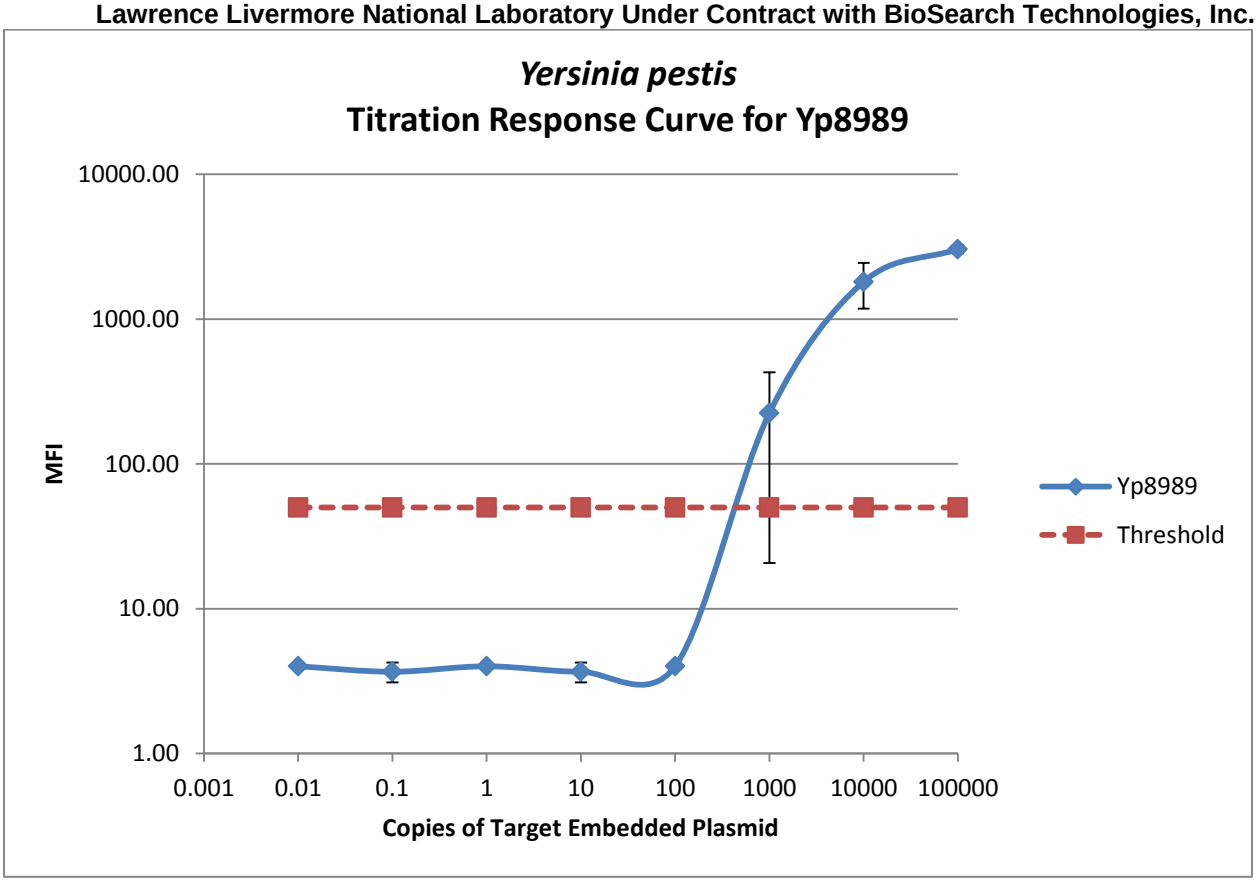

Fig. 12: Titration response curve for Yp4 (8989) signature. Target-embedded plasmids for all *Yersinia pestis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 12 shows a sensitivity of 1000 copies per reaction for the Yp4 (8989) signature.

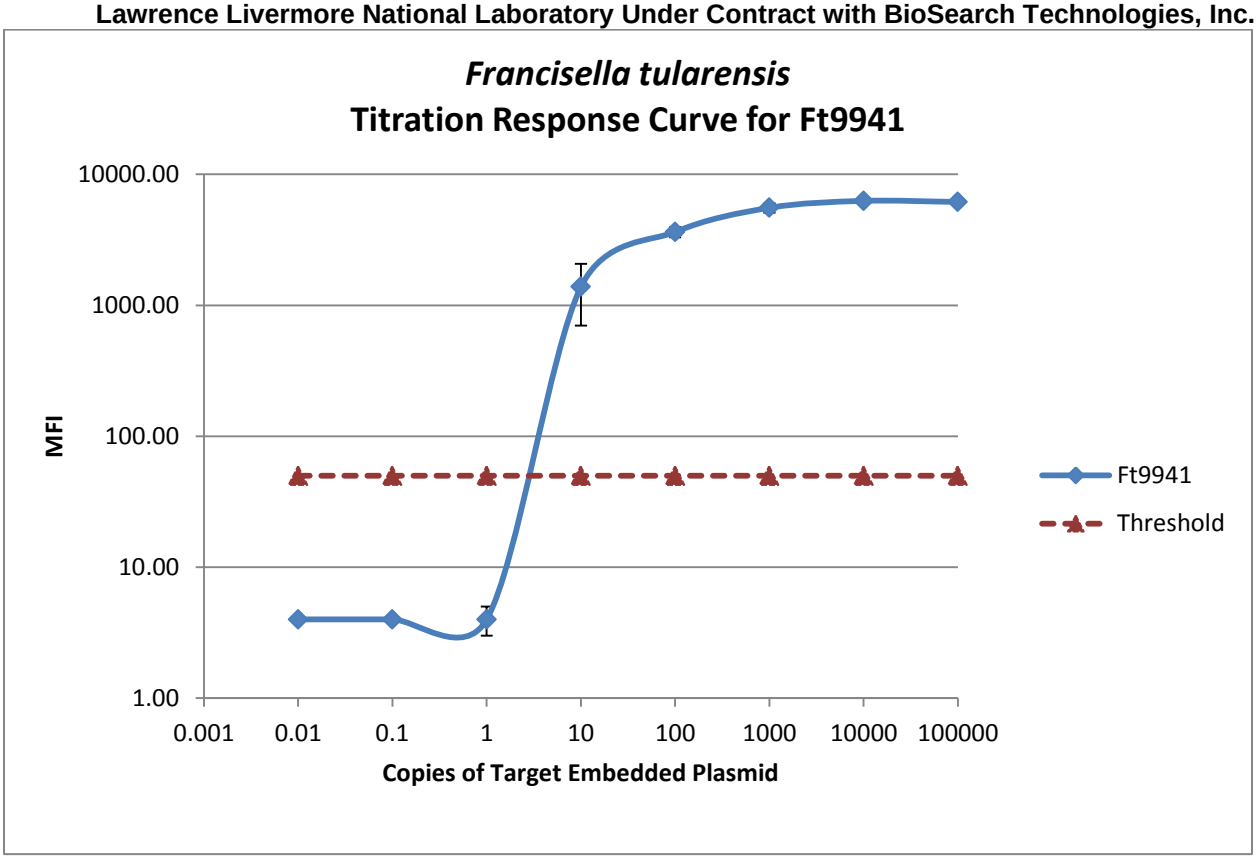

Fig. 13: Titration response curve for Ft1 (9941) signature. Target-embedded plasmids for all *Francisella tularensis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µLto 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 13 shows a sensitivity of 10 copies per reaction for the Ft1 (9941) signature.

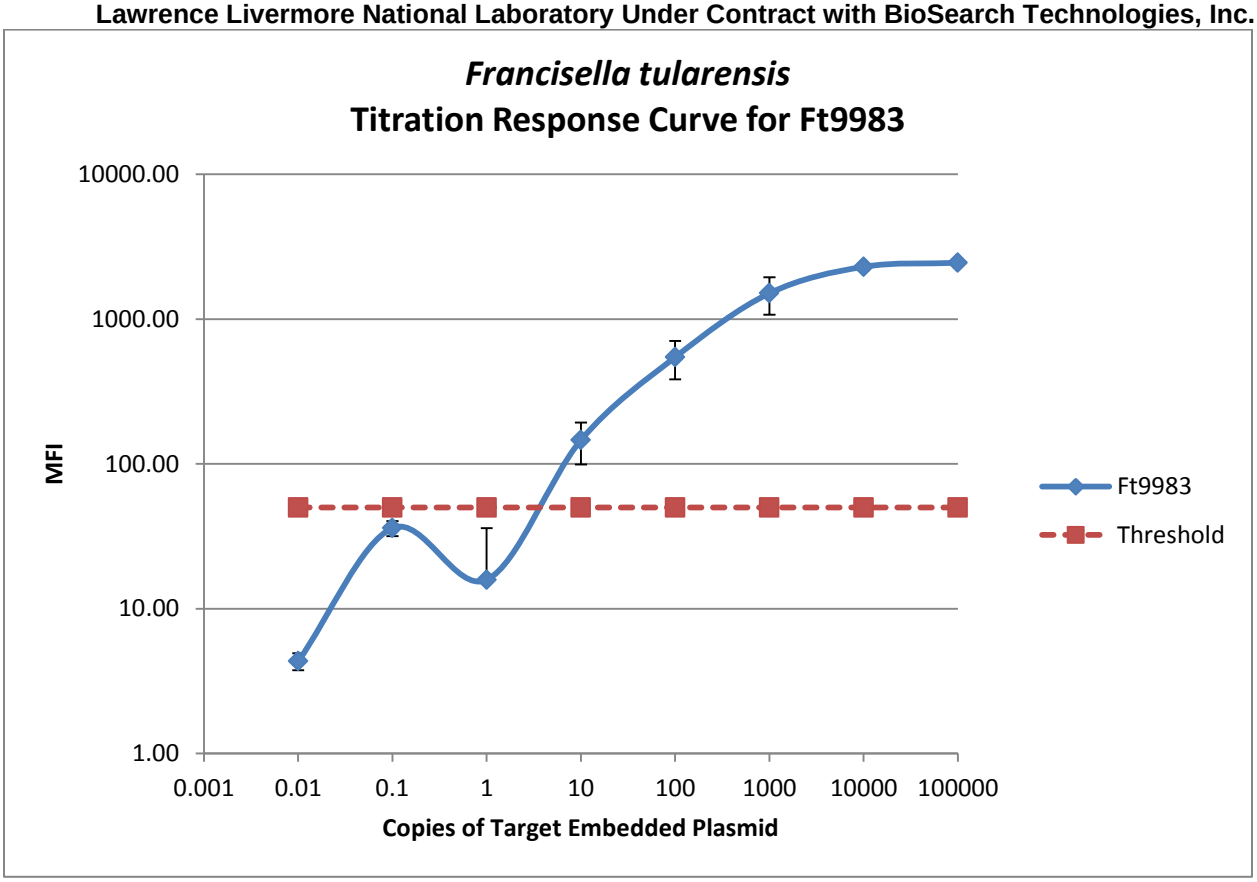

Fig. 14: Titration response curve for Ft2 (9983) signature. Target-embedded plasmids for all *Francisella tularensis* signatures were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 14 shows a sensitivity of 10 copies per reaction for the Ft2 (9983) signature.

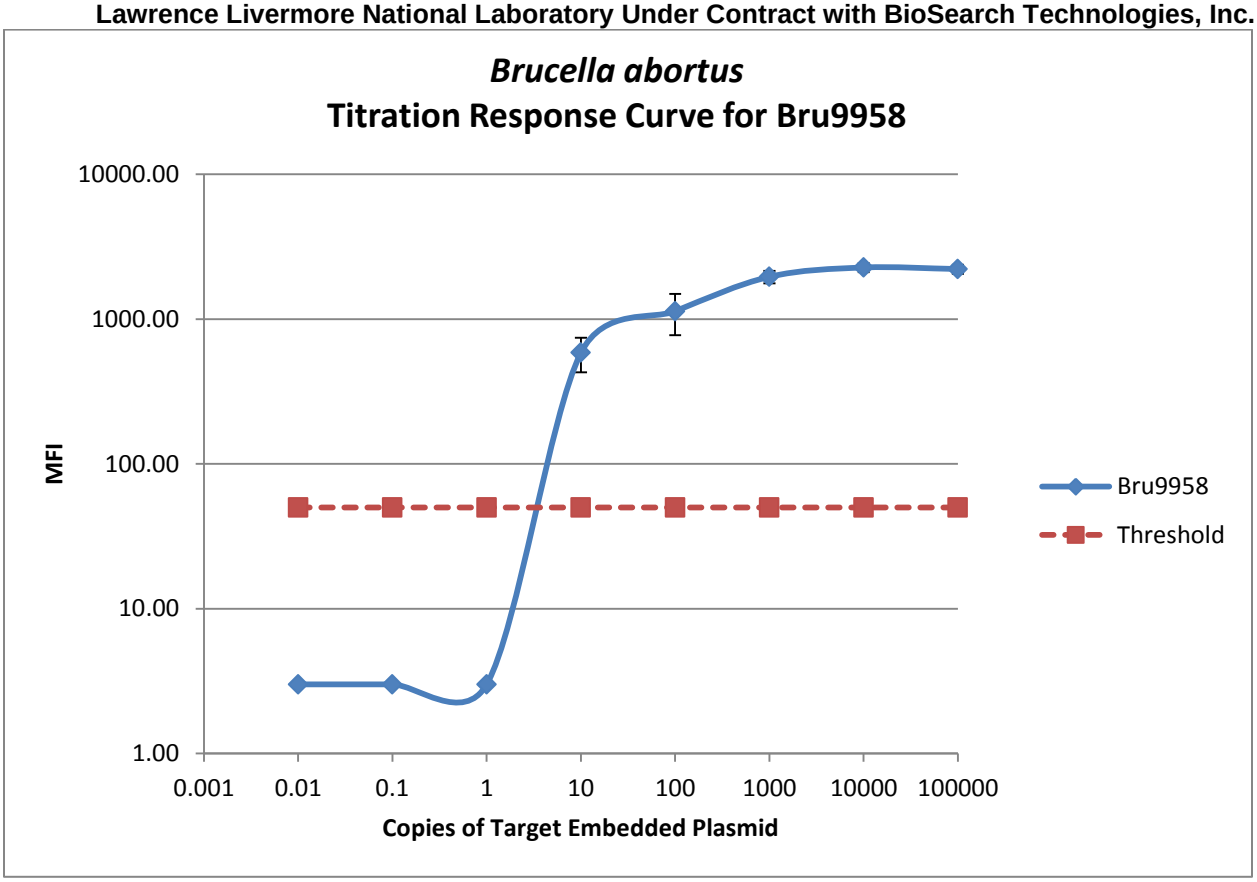

Fig. 15: Titration response curve for Bru1 (9958) signature. Target-embedded plasmids for all *Brucella abortus/melitensis* were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 15 shows a sensitivity of 10 copies per reaction for the Bru1 (9958) signature.

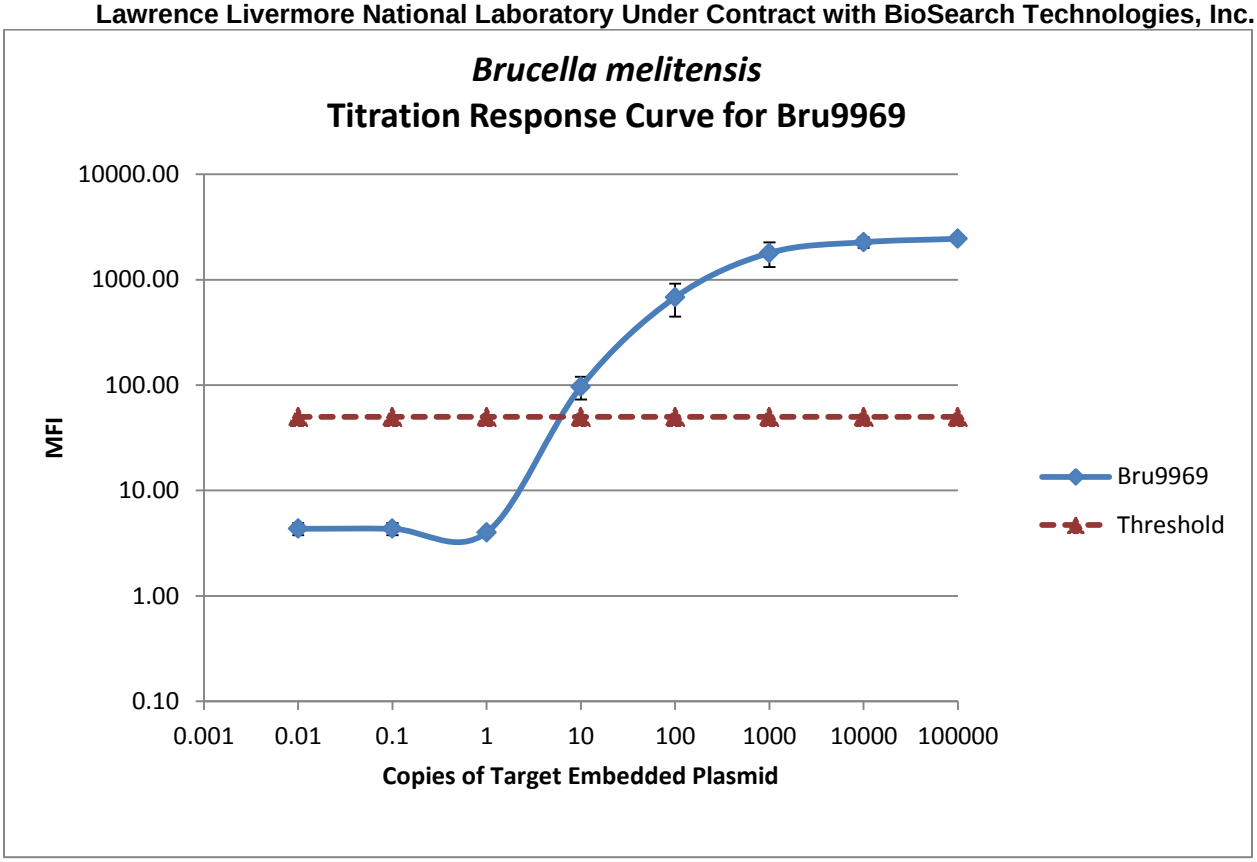

Fig. 16: Titration response curve for Bru2 (9969) signature. Target-embedded plasmids for all *Brucella abortus/melitensis* were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 16 shows a sensitivity of 10 copies per reaction for the Bru2 (9969) signature.

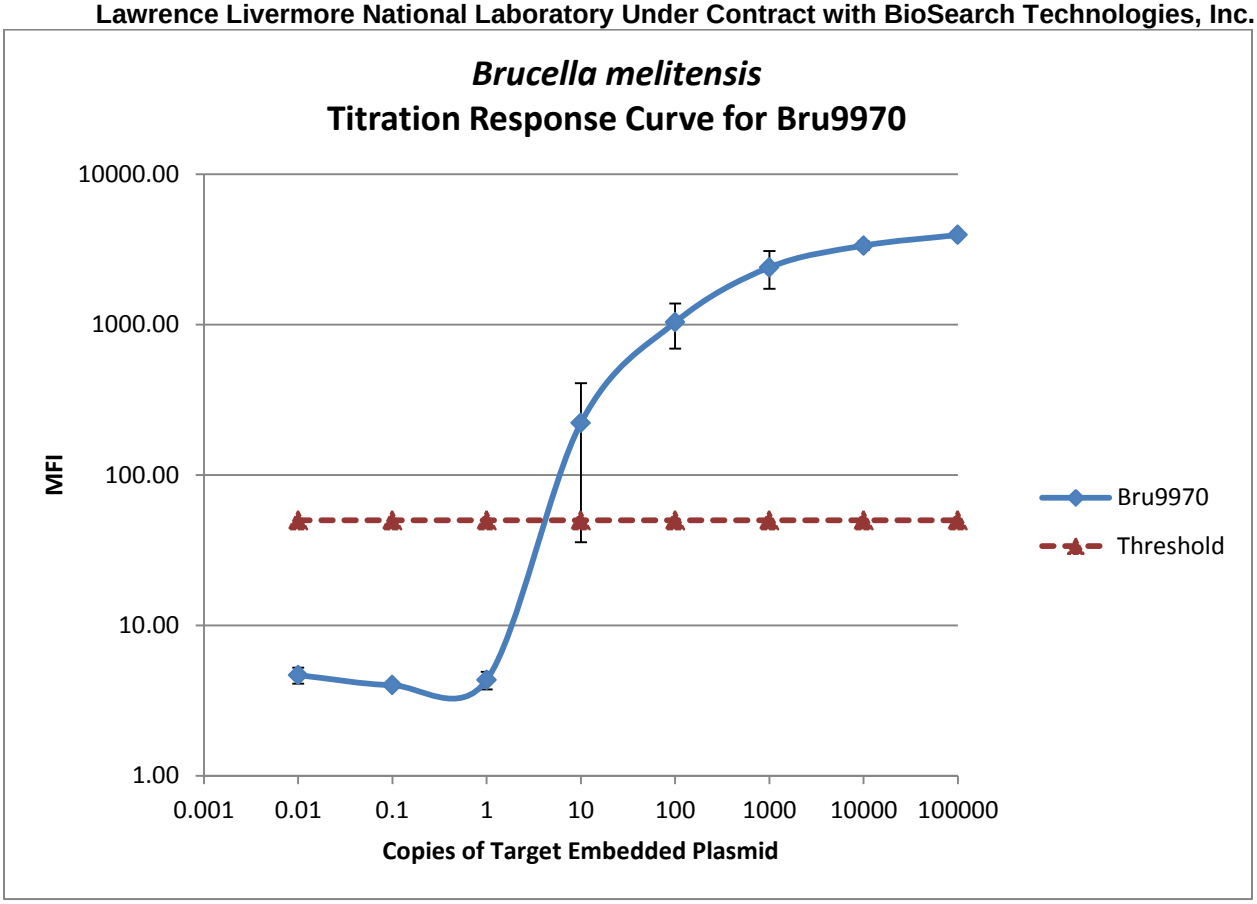

Fig. 17: Titration response curve for Bru3 (9969) signature. Target-embedded plasmids for all *Brucella abortus/melitensis* were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 17 shows a sensitivity of 10 copies per reaction for the Bru3 (9970) signature.

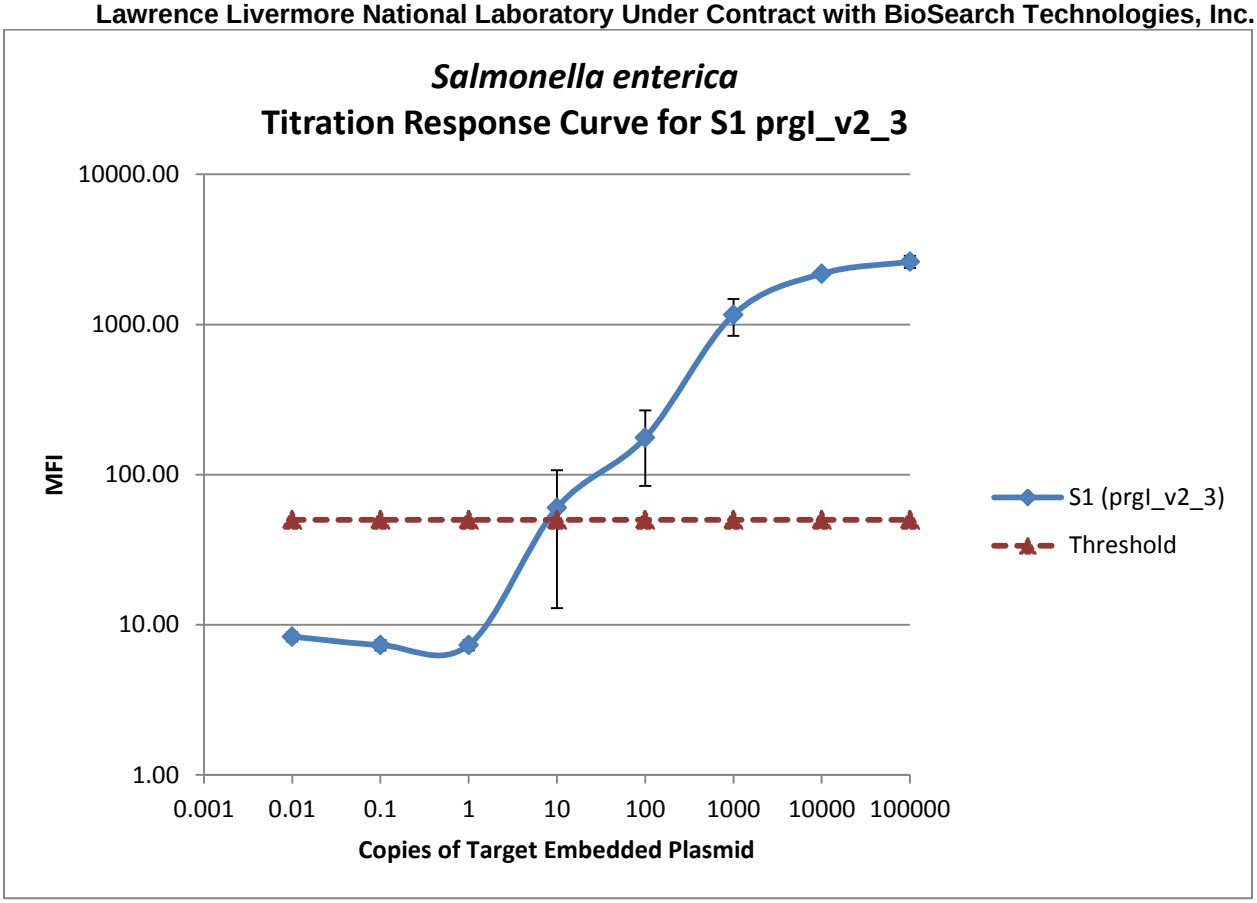

Fig. 18: Titration response curve for S1 (prgI_v2_3) signature. Target-embedded plasmids for all *Salmonella enterica* were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/ reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 18 shows a sensitivity of 10 copies per reaction for the S1 (prgI_v2_3) signature.

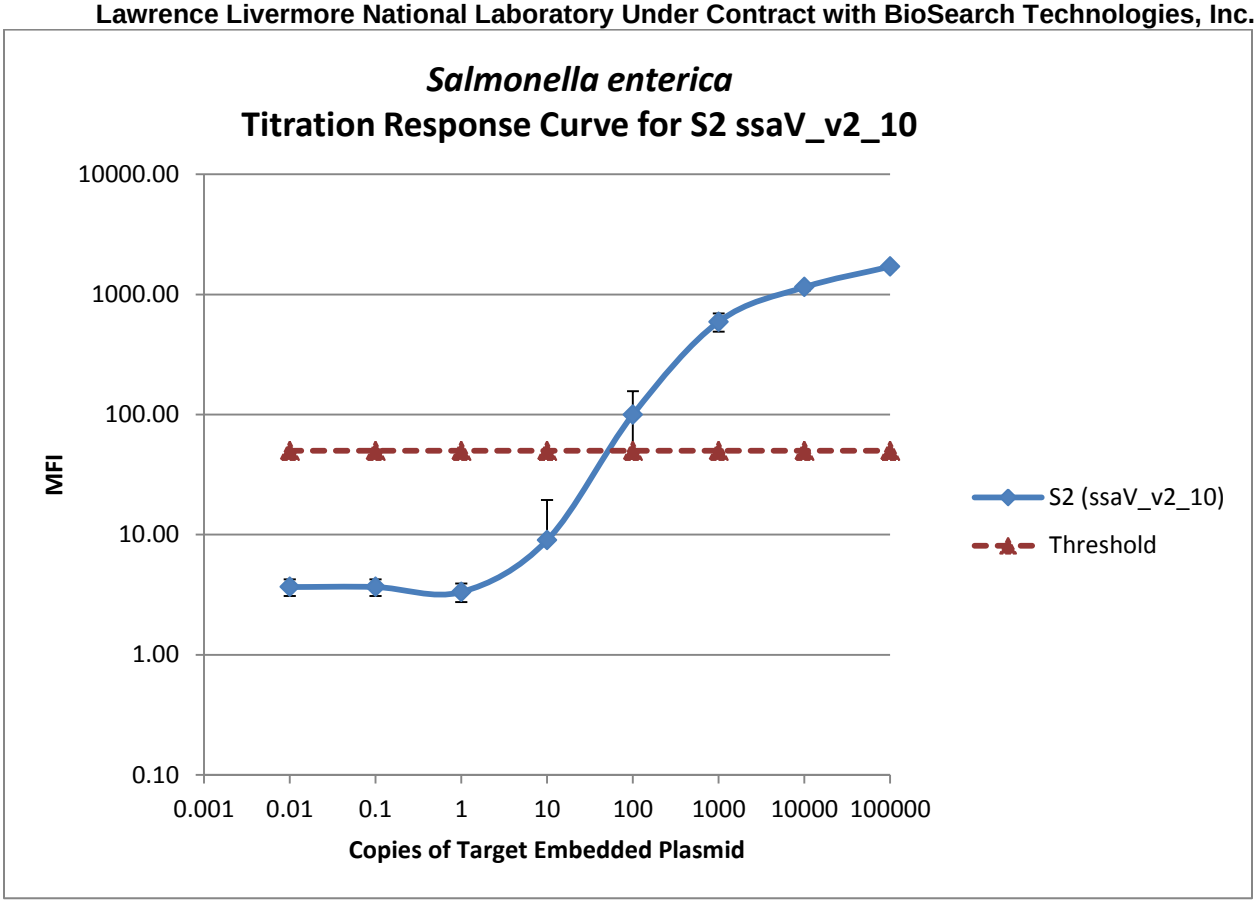

Fig. 19: Titration response curve for S2 (ssaV_v2_10) signature. Target-embedded plasmids for all *Salmonella enterica* were linearized and mixed in equimolar ratios. This material was then titrated over 8 logs from 20,000 copies/µL to 0.002 copies/µL. Triplicate reactions using the multiplex assay were then conducted on each titration point using 5µL of each template titration resulting in a test range of 8 logs from 100,000 copies/reaction. to 0.01 copy/ reaction. A semilog plot was generated with average MFIs used for the plot and standard deviations shown as error bars. Threshold for this signature is shown as a straight dashed red line.

Figure 18 shows a sensitivity of 100 copies per reaction for the S2 (ssaV_v2_10) signature.

In order to ensure that the LODs reported for these assays had sufficient robustness, another set of titrations at and near the initially determined LODs were conducted as before. Here, however, 20 replicates of each reaction were conducted as the final LODs reported had to be within 95% confidence interval (ie: the assay should be expected to deliver at least this LOD 95% of the time or better).

The results of those tests are detailed in tables 16-19 and summarized in Table 1.

Table 17
High Confidence LODs (95% or Greater Probability of Reactivity at LOD target spike)
Francisella tularensis
(*Copies of Target Embedded Plasmid*)

Signature	Target	Average MFI	MFI Range	% Above Threshold	≥ 95% Reactive	LOD
Ft9941	10 copies/rxn	604.13	3-1659	80 (16/20)	N	10 copies/rxn
	100 copies/rxn	3320.35	2368-4618	100 (20/20)	Y	100 copies/rxn
Ft9983	10 copies/rxn	50.65	4-155	35 (7/20)	N	10 copies/rxn
	100 copies/rxn	437.05	129-784.5	100 (20/20)	Y	100 copies/rxn

Table 18
High Confidence LODs (95% or Greater Probability of Reactivity at LOD target spike)
Bacillus anthracis
(*Copies of Target Embedded Plasmid*)

Ba6115	100 copies/rxn	31.25	6-89	15 (3/20)	N	100 copies/rxn
	1000 copies/rxn	271.18	19-622	100 (20/20)	Y	1000 copies/rxn
Ba6132	100 copies/rxn	15.98	5-46.5	0 (0/20)	N	100 copies/rxn
	1000 copies/rxn	117.03	50-353	100 (20/20)	Y	1000 copies/rxn
Ba9485	10 copies/rxn	100.85	3-471	40 (8/20)	N	10 copies/rxn
	100 copies/rxn	613.93	250.5-1529	100 (20/20)	Y	100 copies/rxn

Table 19
High Confidence LODs (95% or Greater Probability of Reactivity at LOD target spike)
Yersinia pestis
(*Copies of Target Embedded Plasmid*)

Yp2040	100 copies/rxn	1249.88	3-2404	75 (15/20)	N	100 copies/rxn
	1000 copies/rxn	4126.95	3320-5400	95 (19/20)	Y	1000 copies/rxn
Yp4683	100 copies/rxn	13.53	5-40	0 (0/20)	N	100 copies/rxn
	1000 copies/rxn	117.90	50-424	95 (19/20)	Y	1000 copies/rxn
Yp5554	100 copies/rxn	63.60	3-256	45 (9/20)	N	100 copies/rxn
	1000 copies/rxn	523.15	260-700	100 (20/20)	Y	1000 copies/rxn
Yp8989	100 copies/rxn	33.45	3-145	20 (4/20)	N	100 copies/rxn
	1000 copies/rxn	248.13	53-481	100 (20/20)	Y	1000 copies/rxn

Table 20
High Confidence LODs (95% or Greater Probability of Reactivity at LOD target spike)
Brucella abortus/melitensis
(*Copies of Target Embedded Plasmid*)

Bru9958	10 copies/rxn	141.95	3-424.5	70 (14/20)	N	10 copies/rxn
	100 copies/rxn	710.95	280-1138	100 (20/20)	Y	100 copies/rxn
Bru9969	10 copeis/rxn	51.33	4-257	40 (8/20)	N	10 copies/rxn
	100 copies/rxn	339.00	147-570	100 (20/20)	Y	100 copies/rxn
Bru9970	10 copeis/rxn	40.00	5-136	25 (5/20)	N	10 copies/rxn
	100 copies/rxn	373.28	96-1132	100 (20/20)	Y	100 copies/rxn

Table 21
High Confidence LODs (95% or Greater Probability of Reactivity at LOD target spike)
Salmonella enterica
(*Copies of Target Embedded Plasmid*)

S1 (prgI_v2_3)	100 copies/rxn	91.88	6-217	70 (14/20)	N	100 copies/rxn
	1000 copies/rxn	567.28	267-1013	100 (20/20)	Y	1000 copies/rxn
S2 (ssaV_v2_10)	100 copies/rxn	42.18	8-85	25 (5/20)	N	100 copies/rxn
	1000 copies/rxn	303.90	194-431.5	100 (20/20)	Y	1000 copies/rxn

Conclusions and next steps:

An 18-plex molecular assay for the detection of *Bacillus anthracis, Yersinia pestis, Francisella tularensis, Brucella abortus, Brucella melitensis,* and *Salmonella enterica* in food matrices has been developed. The signatures comprising the panel have been finalized and an optimized PCR reaction process has been developed.

The assay has been characterized in the laboratory setting with regards to sensitivity, selectivity, and robustness and demonstrated to be effective. Additional testing is necessary in order to complete performance characterization of the multiplex assay in the "field" setting. This testing must be conducted at FDA CFSAN and include tests on actual organism spikes into food matrices.